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SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

ANNUAL and FINAL REPORT

ARTHUR M. DANNENBERG, JR., M.D., Ph.D.

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The paranuclear vacuolization test for injury to human skin. Full-thickness specimens of human skin (1.0 cm^2) were topically exposed to 10 ul of 0.03% to 1.0% SM and organ-cultured for 24 hr at 36 C. There was a straight-line dose-response relationship between the above concentrations of SM and the number of paranuclear vacuoles seen histologically in the epidermis. Within the same SM dosage range, there was also a proportional decrease in ^{14}C-leucine incorporation by the explants.

Early mediators of inflammation produced by SM in human skin. Culture fluids from SM-exposed and control full-thickness human skin explants contained similar amounts of angiotensin-converting enzyme, trypsin-like and chymotrypsin-like proteases, acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, deoxyribonuclease, ribonuclease, interleukin 1 and lactic dehydrogenase. However, the culture fluids from the SM-exposed explants contained increased amounts of histamine, plasminogen activator (PA), and, usually, prostaglandin E_2 (PGE_2), compared to culture fluids from control explants. (continued next page)

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epidermal injury
organ culture
paranuclear vacuoles
^{14}C-leucine incorporation by full-thickness human skin explants
histamine
mast-cell degranulation
prostaglandin E_2
lysosomal enzymes: acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme and lactic dehydrogenase
histochemistry: glucose-6-phosphate dehydrogenase, succinic dehydrogenase, and acid phosphatase
trypsin-like and chymotrypsin-like enzymes
plasminogen activator
deoxyribonuclease and ribonuclease
angiotensin converting enzyme
hydroxyproline
interleukin 1
chemotaxins for macrophages and granulocytes
complement components (C3a, C5a)
RA V

19. ABSTRACT (continued)

After 3 to 4 days in culture, full-thickness human skin explants, exposed to 0.2% SM (but not to 1.0% SM), sometimes showed separation of the epidermis and increased collagenase activity (i.e., hydroxyproline release). Thus, it seems likely that histamine (from local mast cells), and PA and PGE_2 (probably from mast cells and epidermal cells) are some of the early mediators of the inflammatory response of human skin to the topical application of dilute sulfur mustard.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

SUMMARY

Our studies had two purposes: (a) To develop our paranuclear vacuolization test for assessing the amount of injury produced in full-thickness human skin explants by topically applied sulfur mustard (SM), and (b) to identify some of the early mediators of inflammation produced by SM in human skin.

The paranuclear vacuolization test for injury to human skin. Full-thickness specimens of human skin (1.0 cm^2) were topically exposed to 10 ul of 0.03% to 1.0% SM and organ-cultured for 24 hr at 36 C. In this in vitro system, the viable keratinocytes are protected by the same keratinized layer as skin remaining on the donor.

There was a straight-line dose-response relationship between the above concentrations of SM and the number of paranuclear vacuoles seen histologically in the epidermis. Within the same SM dosage range, there was also a proportional decrease in ^{14}C-leucine incorporation by the explants. Thus, the number of paranuclear vacuoles reflected the decrease in protein synthesis by injured epidermal cells.

The epidermis of full-thickness untreated (control) human skin explants usually remained viable for 7 days when stored at 4 C in culture medium. During storage, a relatively small number of paranuclear vacuoles developed within the epidermis, but these explants were still quite satisfactory for testing SM toxicity. Incubation (for 4 or 24 hr at 36 C) of such skin explants reduced (often by 50%) the small number of paranuclear vacuoles produced during 4 to 7 days of storage. This reduction was probably caused by autolysis of many of the vacuolated cells.

Two types of paranuclear vacuoles could be identified by both light and electron microscopy: a storage-type and a toxicant-type. The storage-type seemed to be caused by autolysis of cell components. The toxicant-type seemed to be caused by an invagination of the plasma membrane. Only toxicant-type vacuoles appreciably increased in number when skin explants were exposed to mustard or other toxicants.

Early mediators of inflammation produced by SM in human skin. Mediators released from SM-injured human skin that initiate the inflammatory response have not been adequately identified. Organ culture of full-thickness skin explants enables us to do so, because injury to the skin can be made in vitro, eliminating the rapid leakage of serum and infiltration of leukocytes that occurs in vivo. Organ-culture fluids were then assayed for various inflammatory mediators.

We found that the culture fluids from SM-exposed and control explants contained similar amounts of angiotensin-converting enzyme, trypsin-like and chymotrypsin-like proteases, acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, deoxyribonuclease, ribonuclease, interleukin 1, and lactic dehydrogenase. However, the culture fluids from SM-exposed explants contained increased amounts of histamine, plasminogen activator (PA), and, usually, prostaglandin E_2 (PGE_2), when compared to culture fluids from control explants. After 3 to 4 days in culture, full-thickness human skin explants, exposed to 0.2% SM (but not to 1.0% SM), sometimes showed separation of the epidermis and increased collagenase activity (i.e., hydroxyproline release).

Thus, histamine (from local mast cells), and PA and PGE_2 (probably from mast cells and epidermal cells) are some of the early mediators of the inflammatory response of human skin to the topical application of dilute sulfur mustard.

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INTRODUCTION

Statement of Problem under Study

Our first mission was to develop a practical *in vitro* test for chemical substances, such as sulfur mustard (SM), that are toxic to *human* skin. Such a test would eliminate the injury and discomfort created by skin-testing a person with toxic substances, and also eliminate all systemic absorption of the toxicant. Isolated human keratinocytes (in culture) can be used for testing the effects of various toxicants (1-5). However, because they are usually not protected by a keratinized layer, such free cells are more suceptible to injury than cells remaining in place in the intact skin. Full-thickness human skin explants in organ culture more closely match the *in vivo* situation: Similar to skin remaining on the host, such full-thickness skin explants can be topically exposed to many disinfectants and organic solvents with no apparent harm.

Our second mission was to identify, for the future development of new therapies, some of the early mediators of inflammation produced by SM in human skin.

Background and Rationale

In a previous study (6), we developed the paranuclear vacuolization test to evaluate effects of topically applied sulfur mustard (SM) and other toxicants on full-thickness guinea pig and rabbit skin explants. In the present study, we applied this test to *human* skin. Included is information on how long human skin could be kept at 4 C and still remain usable in our paranuclear vacuolization test for toxicity. The information provided should enable a wider use of the paranuclear vacuolization test for assessing the toxicity of topically applied chemical agents. It also should facilitate the development of protective ointments and decontaminating procedures for sulfur mustard.

Injury to skin causes an inflammatory response. The *initial mediators* of this process are derived from local cells. The response is then amplified by mediators from the extravasated serum and infiltrating leukocytes. The serum releases phlogistic components of the complement, kinin, plasmin, and clotting systems (7-11). The leukocytes (and local cells) release various cytokines, prostaglandins, leukotrienes, proteases, leukckinins, and other mediators (7-11).

Full-thickness human skin explants were exposed topically to SM, and were then organ-cultured in serum-free medium. The culture fluids extracted the inflammatory mediators from the explant, so that they could be assayed. Extravasated serum and infiltrating leukocytes were almost completely absent in these explants. This *in vitro* culture system was, therefore, quite suitable for identifying some of the stable mediators that initiate the inflammatory response.

Recently, there have been many studies on the various cytokines produced by cells and tissues (12). A complete study of cytokines as initiators of the inflammatory response remains to be performed, but it has been suggested that keratinocytes have the ability to release IL-1 (13). The organ culture system described herein may not be satisfactory for the identification of such cytokines, because many of them are locally produced and short-lived. The use of in situ hybridization to visualize the mRNA of the various cytokines will undoubtedly make a considerable contribution, because the mRNA "factory" seems to be much more stable than its product. Thus, these studies on the early inflammatory mediators represent only a beginning, and other early mediators are bound to be discovered in the next few years.

EXPERIMENTAL METHODS

Organ culture of 1.0-cm^2 human skin specimens (6,14,15)

The skin specimens were discards from a variety of surgical operations (16). They were placed on a sterile plastic sheet, made wet with Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY, Cat. No. 310-4025) containing penicillin (1000 U/ml) and streptomycin (1000 ug/ml), and the subcutaneous fat was removed with scissors. Trimming of the specimens was performed in a hood, and gloves and a surgical mask were worn. The specimens were used immediately or stored overnight in the supplemented RPMI 1640 (described below).

For organ culture, the skin was cut precisely into 1.0-cm^2 full-thickness pieces and washed three times with the antibiotic-containing Hanks' solution. Then, each explant was placed in a small, sterile, plastic Petri dish (35 x 10 mm, Falcon Plastics, Division of Becton Dickinson Co., Oxnard, CA). The epidermis was patted dry with sterile surgical gauze, and two drops of RPMI 1640 culture medium were added to the Petri dish in order to keep the base of the explant moist. Then, in a stainless steel hood with a draft of 150 linear feet of air per minute, we spread (by means of a Hamilton syringe) 10 ul of dilute SM (or its vehicle) over the entire upper surface of the explant. Our standard concentration of SM was 1.0% in methylene chloride ($MeCl_2$), but more dilute concentrations were also used for some of the studies herein reported. The specimens were left in the hood for 30-40 min at room temperature before they were organ-cultured.

Certain skin specimens were similarly exposed to 5% nitrogen mustard (HN2), dissolved in equal parts of $MeCl_2$ and dimethyl sulfoxide (DMSO). Nitrogen mustard hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO 63178). When the HN2·HCl was neutralized with concentrated ammonia, an NH_4Cl precipitate formed and the HN2 dissolved in the organic solvents. 5% HN2 in $MeCl_2$-DMSO produced lesions similar to those produced by 1% SM.

Our culture medium was RPMI 1640 (2.0 or 2.5 ml/Petri dish) (GIBCO Laboratories, Cat. No. 320-1875), supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and additional glutamine (2.0 mM). (The final concentrations are in parentheses.) Whenever the culture fluids were to be assayed for hydroxyproline, Dulbecco's Modified Eagle Medium (GIBCO, Cat. No. 320-1885) was substituted for the RPMI 1640, because the former does not contain OH-proline. For the ^{14}C-leucine experiments, ^{14}C-leucine (0.25 uCi/ml, 350 mCi/mmol specific activity) was added to the culture medium.

Three small Petri dishes, each containing an explant, were then placed in one large Petri dish (100 x 15 mm, Falcon Plastics), and the large Petri dishes were stacked in a heavy plastic vacuum jar (Oxoid U.S.A., Columbia, MD). The jar was gassed with a 95% O_2--5% CO_2 mixture at 1.2-1.3 atmospheres of pressure, and then was sealed. It was rocked six times per minute in an incubator at 36 C for 24 hr. The tops of the 1.0-cm^2 skin explants were not covered by the culture medium, but were exposed directly to the gaseous O_2/CO_2 mixture. They did, however, become moist.

The fixation, and the preparation of Giemsa-stained 1- to 2-um glycol-methacrylate-embedded tissue sections from these explants are described in detail in references 6, 14, and 16.

Comment on the organ-culture technique. The question arose whether intact full-thickness skin explants would release more inflammatory mediators if they were placed with a freer flow of medium beneath them. Therefore, four 1-cm^2 full-thickness explants from 1-day SM lesions produced in vivo in a rabbit (14) were cultured resting on non-toxic stainless steel grids with opposite edges (2 mm wide) bent at right angles. Four similar explants were also cultured resting on a nylon mesh support, and four explants were cultured without any support. After 24 hr in culture at 36 C, the culture fluids were collected, centrifuged (to removed the few cells and particles present), and assayed for total protein (14), β-galactosidase (17,18), and lactic dehydrogenase (18,19). Similar levels of protein and the two enzymes were found in culture fluids from all three types of preparation. Normal (rabbit) skin explants cultured in the same manner confirmed these findings.

Counting of paranuclear vacuoles

Counting of paranuclear vacuoles in human skin explants is not as precise as counting vacuoles in animal skin explants. In general, human epidermis is much thicker than animal epidermis, and the size and shape of the vacuoles are more variable.

Figure 1 shows representative paranuclear vacuoles, which can be used to guide one's counting. Vacuoles were counted if they were 2 um or larger and were next to the nucleus. If the cell had a pyknotic nucleus and a vacuole, we did not count that vacuole. Narrow slits beside nuclei were not counted, because they might have been merely shrinkage artifacts. Almost all vacuolated epidermal cells contained only one vacuole per cell.

The vacuole counts were adjusted if the epidermis in the tissue section was less than, or more than, 1.0 cm in length. In other words, to determine the number of paranuclear vacuoles per cm of skin, we measured the length of the tissue section in which the vacuoles were counted.

For commercial use, vacuole counting could be performed by an image analyzer. In this case, we recommend that all vacuoles above 2 um in size be counted, whether or not the vacuole is in a pyknotic cell.

Viability of the explants

According to Dame Honor B. Fell, of Strangeways Laboratories, Cambridge, England, (personal communication) histologic evaluation with light microscopy is one of the best ways of determining cell and tissue viability in organ-cultured explants: As a basal keratinocyte dies, its nucleus and cytoplasm appear more homogeneous, and its cellular boundaries become indistinct. Using these properties as indicators of viability, we found that the entire epidermis of full-thickness human skin explants almost always survived well in organ culture for 24 hr at 36 C, and that the basal epidermal cells and hair follicle cells often survived well for 3 or 4 days at 36 C.

Electron microscopy (20)

Pieces (2- to 3-mm thick) were cut from the center of the skin explant and fixed for 4 hr at 4 C in 2% paraformaldehyde--2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). They were transferred to 0.1 M cacodylate

buffer (pH 7.2) (containing 0.3 M sucrose) and left overnight at 4 C. They were then trimmed to about 1.5 mm and postfixed in 1.0% (cacodylate-buffered) osmium tetroxide for 90 min, rinsed in the buffered sucrose solution, dehydrated in alcohols, embedded in Spurr's epoxy formulation (21), cut on an ultramicrotome, and stained with lead citrate (22).

Measurement of ^{14}C-leucine incorporation into proteins of the skin explants

Details of this method are published in reference 6. In brief, full-thickness skin explants were organ-cultured for 24 hr at 36 C in medium RPMI 1640, containing ^{14}C-leucine, as described above in the "Organ Culture" section. After incubation, the radioactive medium was decanted, and each explant was washed, minced, and homogenized. The homogenate was precipitated with trichloroacetic acid (TCA) and centrifuged. The pellet was washed 5 times with TCA and once with a lipid solvent. Then, it was digested sequentially with NaOH and Protosol (Du Pont-New England Nuclear Research Products, Boston, MA), and the amount of ^{14}C-radioactivity was read in a scintillation counter.

Histamine

The histamine assays were performed in the laboratory of Dr. Carol A. Hirshman by the fluorometric method originally described by Shore (23) and modified by Siraganian (24,25). The sample containing histamine was diluted with NaCl(0.9%)-EDTA-detergent (Brij-35) solution. Saturated (30%) NaCl and 1-butanol were added, and the mixture was alkalinized to extract the histamine into the butanol. The butanol (top) layer was collected, acidified with 0.1 n HCl, and extracted with 1-heptane. (These procedures removed the histamine from interfering substances.) The aqueous phase was collected and alkalinized. o-Phthalaldehyde was added, which forms fluorescent histamine-o-phthalaldehyde at alkaline pH. The histamine-o-phthalaldehyde reaction was stopped with phosphoric acid, and then the fluorescence was read with an activating wavelength of 350 nm and a fluorescent wavelength of 450 nm in a recording fluorometer.

This whole procedure is automated in an apparatus composed of modules from the Technicon Corp. Tarrytown, NY, assembled by Alpkem Corp., Clackamas, OR. In this apparatus, up to 30 samples per hour can be analyzed for histamine. A linear relation exists between histamine concentration and the fluorescence in the histamine range of 0 to 25 ng per ml.

Prostaglandin E_2

PGE_2 was assayed with the AMI PGE_2 Enzyme Immunoassay Kit with BioMag Magnetic Separation (AMI Research Products, Cambridge, MA 02138, Code No. 6101). The assay is based on the competition of variable amounts of PGE_2 in samples with a fixed amount of alkaline phosphatase-labeled PGE_2 for a limited number of binding sites on a rabbit anti-PGE_2 antibody. With more unlabeled PGE_2 in the sample, less labeled PGE_2 is bound to the specific antibody. The antibody-bound analyte is separated from the unbound analyte with magnetic goat anti-rabbit antibody and centrifugation. The resulting magnetic pellet containing the alkaline phosphatase-labeled PGE_2 is reacted with the substrate, para-nitrophenyl phosphate, and the absorbance at 405 nm is determined. Absorbance is correlated with concentration by means of a standard curve.

Lysosomal enzymes, lactic dehydrogenase (LDH) and total protein

Culture fluids from 1.0-cm^2 full-thickness SM-exposed human skin explants, along with culture fluids from the appropriate control explants, were assayed for acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, LDH, and total protein. Briefly, the first three hydrolases were assayed with p-nitrophenyl phosphate, p-nitrophenyl β-glucuronide and p-nitrophenyl β-galactopyranoside as the substrates, respectively (17,18). The release of nitrophenol was measured at 410 nm. Lysozyme was assayed by the lysis of Micrococcus lysodeikticus in agar plates (26-29). LDH was assayed by the reduction of pyruvate in the presence of reduced β-nicotinamide adenine dinucleotide (NADH) (18,19). LDH is L-lactate: NAD oxidoreductase, an enzyme that favors the formation of lactate and NAD over pyruvate and NADH (19). The total protein was assayed by Coomassie blue binding (14,30).

Histochemistry of acid phosphatase, glucose-6-phosphate dehydrogenase and succinic dehydrogenase

Acid phosphatase activity was visualized in unfixed frozen sections by incubating at 23 C for 4 hr with the Naphthol AS-BI phosphate substrate of Burstone (31-33) (in acetate buffer at pH 5.2 containing 0.2% $MnCl_2$), and Fast Red Violet LB salt as the simultaneous diazonium coupling agent.

Glucose-6-phosphate dehydrogenase (G6PD) and succinic dehydrogenase were visualized in unfixed frozen sections with nitro blue tetrazolium (nitro BT) (34,35). The sections were incubated for 1 to 4 hr at 36 C in a solution containing 1.0 M glucose-6-phosphate or 1.0 M sodium succinate (0.4 ml), phenazine methosulfate (1.0 mg), nicotinamide adenine dinucleotide phosphate (NADP) (3 mg), and 2.0 ml of nitro BT stock solution (35), containing 0.14% nitro BT, 0.01% NaCN, 0.01 M $MgCl_2$ in 0.05 M Tris buffer at pH 7.3.

Proteases

Culture fluids from 1.0-cm^2 full-thickness human skin explants, topically exposed in vitro to SM, were assayed for trypsin-like and chymotrypsin-like proteases with the synthetic peptide substrates, L-leucyl-glycyl-L-arginyl-aminofluorocoumarin (LGA-AFC) (36-39) and N-benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN) (39-41).

Plasminogen activator

A most sensitive and specific plasminogen activator assay is the fibrin-plate method of Edward Reich's group (42), used by the Gerald S. Lazarus group at the University of Pennsylvania (43,44). Dr. Pamela J. Jensen of the latter group guided us in establishing the method in our own laboratory.

Briefly, ^{125}I-fibrinogen was prepared (43-45) and about 0.1 uCi of it was dried in each well of a 96-well plastic sheet. Just before use, fibrin was formed in the wells with thrombin (1 U/ml, 300 ul per well). The wells were gently washed, and the skin explant culture fluids were added in duplicate (with and without commercial plasminogen). After the appropriate incubation at 37 C, the supernatant fluids (200 ul) were harvested and their radioactivity measured in a

gamma counter. The total radioactivity released by trypsin was also measured. Standard curves were made with commercial urokinase, with and without the addition of plasminogen.

Hydroxyproline

The OH-proline in culture fluids from full-thickness 1.0-cm^2 human skin explants was measured by the method of Berg (46). The soluble peptides were hydrolyzed by heating the fluids with an equal quantity of 12 N HCl in an oven at 105 C for 18 hr, and the acid was neutralized with NaOH. The samples were mixed with chloramine-T (dissolved in 2-methoxyethanol). Then, thiosulfate was added, and the interfering substances were extracted with toluene. The tubes were heated for 30 min in a boiling water bath and cooled. Heating in the presence of chloramine-T and thiosulfate oxidizes OH-proline to pyrrole.

The pyrrole was extracted in toluene and mixed with a solution containing Ehrlich's reagent. After exactly 25 min at 23 C, the optical densities were read at 560 nm. The Ehrlich's reagent solution is p-dimethylaminobenzaldehyde in ethanol and sulfuric acid. It reacts with the pyrrole to form a chromaphore. Standard solutions of OH-proline were included as controls.

Deoxyribonuclease (DNase) and ribonuclease (RNase)

DNase and RNase in (18- to 24-hr) culture fluids from full-thickness 1.0-cm^2 human skin explants were assayed with salmon testes DNA (Sigma Chemical Co., Cat. No. D-1626) and yeast RNA (Sigma, Cat. No. R-6750) as the substrates (47). $LaCl_3$ in an acidified water-ethanol solution was used as the precipitating agent. After centrifugation, the optical density of the supernatant fluid was read in an ultraviolet spectrophotometer at 260 nm.

Angiotensin-converting enzyme (ACE)

This enzyme is released when vascular endothelial cells are damaged (48). SM is known to damage dermal microvasculature because it produces a markedly edematous response.

The fluorimetric assay of Carmel et al. (49,50) was used. Ortho-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (ABz-Gly-Phe(NO_2)-Pro) was obtained from Sigma Chemical Co. (Cat No. A-4408). After dissolving in 0.4 ml of methanol, it was made into a 1.16 mM stock solution (14 mg in 25 ml 0.2 M Tris-HCl buffer (pH 8.2) containing 1.0 M NaCl). This stock solution was diluted 1:1 in the same Tris-NaCl buffer for the ACE assay, or 1:1 with 0.1 M disodium ethylenediamine-tetraacetate (EDTA) solution (in the Tris-NaCl buffer), which serves as a zero time control. (ACE is a zinc-requiring enzyme. The removal of zinc, by EDTA, from the skin culture fluids stops ACE activity.)

Assay procedure. Full-thickness human skin explants (1.0 cm^2) were exposed topically in vitro to 1% SM or its diluent $MeCl_2$ and incubated for 3 to 20 hr in RPMI 1640 in an atmosphere of 95% O_2--5% CO_2, as described above. The culture fluids were centrifuged and stored at -70 C until assayed for ACE.

For this assay, these fluids were thawed and added in 100-ul amounts to either 200 ul of the preincubated substrate solution or 200 ul of the preincubated zero-time control solution. After (usually) 45 min of incubation at 40 C, the reaction was stopped by the addition of 3.0 ml of 0.1 M EDTA in the Tris-NaCl buffer. The fluorescence was read in a 4.2-ml cuvette (1.0 cm across) at room temperature in a Perkin-Elmer fluorescence spectrophotometer at a 360 nm excitation wavelength and a 410 nm emission wavelength. The fluorescent intensity was normalized using a standard solution of 2.5×10^{-7} M quinine sulfate in 0.1 M H_2SO_4.

The difference between the fluorescence in the substrate solution and in the substrate-EDTA solution (zero-time control) is a measure of the units of enzyme activity. The units can be converted to micromoles of amino-benzoylglycine (ABz-Gly) released from the substrate by means of a quinine sulfate standard (see 49). One unit is equivalent to 1.5 nmol of ABz-Gly in the 3.3-ml sample read in the fluorometer. Quinine has a fluorescence 5.4 times that of ABz-Gly (49). (Under our experimental conditions, the quinine standard (3.3 ml) shows a fluorescence 3.0 units greater than its solvent (0.1 M H_2SO_4).)

Interleukin 1 (IL-1) released in vitro by full-thickness skin explants

Keratinocytes produce IL-1 in response to injury (13). Therefore, the culture fluids from SM-exposed and control full-thickness human (and rabbit) skin explants were assayed for IL-1 by Dr. Gregory Beck in Dr. Gail S. Habicht's laboratory, at the State University of New York at Stony Brook, by the thymocyte proliferation assay (51). Thymocytes from 4- to 8-week-old BALB/c or C3H/HeJ mice were suspended in culture medium RPMI 1640 containing 10% calf serum, 1% antibiotics, 1% nonessential amino acids, 1% glutamine, and 5×10^{-5} M 2-mercaptoethanol. The thymocytes were cultured for 72 hr at 5×10^5 cells/well in Falcon (96-well) flat-bottomed tissue culture plates in the presence or absence of 0.3 ug/ml Concanavalin A (Con A). For the final 8 hr of incubation, 1 uCi ^{3}H-thymidine (6.7 Ci/mmol) was added along with various dilutions of the samples to be assayed for IL-1 activity. Then, the cells were collected on glass fiber filter paper with an automatic harvester, added to vials containing 4 ml of scintillation fluid, and their radioactivity was measured in a well-type beta-scintillation counter. The results were expressed as the increase in disintegrations per minute (dpm). This increase represents the ^{3}H-TdR incorporation due to IL-1, i.e., the dpm of the thymocyte preparation containing Con A plus the culture fluid sample minus the dpm of the thymocyte preparation containing only Con A.

Assay of chemotactic activity for polymorphonuclear granulocytes (PMN) and for mononuclear phagocytes (MN), i.e., monocytes and macrophages

Dr. Donald L. Kreutzer (52,53), of the University of Connecticut School of Medicine, and Dr. Ralph Snyderman (54), of Duke University School of Medicine, advised us on setting up these methods.

Culture fluid (from SM-exposed or control skin explants) (110 ul for MN and 115 ul for PMN) was added in the lower compartment of a blind-well chemotaxis chamber (Neuro Probe, Inc., Bethesda, MD 20034, Cat. No. 100-187). (The sizes of the upper and lower compartments were 200 ul and 100 ul, respectively.) Upon the convex meniscus of the filled lower compartment, a Millipore (55) or Nuclepore (54) filter was carefully placed, avoiding bubbles. For PMN, 3-um-pore-size Millipore filters, (Millipore Corp., New Bedford, MA 01730, Cat. No. SC9Q018A3),

cut to about 12 mm in diameter with a cork borer, were used. For MN, 5-um-pore-size, polyvinyl pyrrolidone-coated Nuclepore filters, 13 mm in diameter, (Nuclepore Corp., Pleasanton, CA 94566, Cat. No. NMF-5) were used. The Nuclepore filter was placed on the meniscus with the dull side up, but the two sides of the Millipore filter are not appreciably different.

After the top half of the Boyden chamber was screwed down tightly, 0.20 ml of the PMN or MN cell suspension (see below) was pipetted into the upper compartment. At 37 C, in humidified air containing 5% CO_2, the chambers were incubated 45 min for PMN and 90 min for MN.

For PMN, the cell migration was stopped by partly submerging the chambers in a cracked ice bath. Then, the cell suspension in the upper compartment was removed by one rapid shake, and the chamber was disassembled. The PMN-containing filters were placed for 1 to 5 min in 100% ethanol without prior rinsing. After fixation, the filters were rinsed in tap water for about 5 min and stained for 5 to 15 min with alum hematoxylin (Accra Lab, Bridgeport, NJ 08014, Cat. No. 56007). They were then rinsed in tap water and dried overnight, between two layers of filter paper (Whatman #3, qualitative, 24 cm in diameter), pressed down with heavy textbooks.

A thin plastic rack, containing 60 two-ml wells to hold the filters, was cut from a 96-well sheet. Four holes (3 to 4 mm in diameter) were cut in the sides of each well, and one hole was cut in the base (6 mm in diameter). This holding rack, placed in a shallow stainless steel tray, was used for the ethanol fixation, and for the rinsing and staining procedures.

The air-dried, stained filters were cut with a 6 mm-Baker skin biopsy punch (Baker-Cummins Division of Key Pharmaceuticals, Inc., Miami, FL 33169). (The central areas, containing the cells, were only 4.7 mm in diameter.) Then, the filters were mounted on a standard, 1- x 3-inch, microscope slide in cedarwood oil (Fisher Scientific Co., Silver Spring, MD 20910, Cat. No. 0-40), in 3 rows, each containing up to 6 filters. For this purpose, two 3-mm strips of paper masking tape were placed near the edge of the slide. The oil was dropped on top of each filter. Flo-Texx Mounting Medium (Lerner Laboratories, Stamford, CT 06902, Cat. No. M770-1) was placed outside the masking tape strips and a 50 x 22 mm coverslip placed over everything. The Flo-Texx sealed the coverslip in place, but was not allowed to mix with the oil because such a mixture becomes cloudy. Immersion oil may be used instead of cedarwood oil, but with immersion oil, cloudiness sometimes occurs in a few days.

For MN, no chilling in the ice bath was used. Instead, we quickly placed the MN-containing filters, dull side down, on a microscope slide and air-dried them at 23 C. Then, we fixed the cells to the filters by applying 1 drop of 100% ethanol and allowing it to evaporate at 23 C. The filters were stained for 20 min with a dilute, buffered Giemsa solution: 2.0 ml of Giemsa Stain (Harleco, Gibbstown, NJ 08027, Cat. No. 620) mixed with 15 ml of 0.02 M sodium phosphate buffer (pH 6.6). They were rinsed by gentle dipping into a large beaker of distilled water. Then, they were dipped in a 0.35% $NaHCO_3$-2% $MgSO_4$ solution for about 30 sec, and air-dried. The Nuclepore filters were then mounted in Flo-Texx.

With MN, all the cells in 5 high power fields (HPFs) (40 X objective lens, 500 X magnification) on the shiny surface of each filter were counted, using the ocular grid described below in the area described below. Our chemotaxis index was the total number of MN in 5 HPFs.

With PMN, counting was more complex, because the method measured the degree of leukocyte penetration into the Millipore filter (55). First, the top of the filter was surveyed for uniformity (clumping was rare). Then, 5 representative grid areas were chosen and counted from the center third of the filter (usually the top, bottom, right, left, and center of this central third). A 40 X objective and 12.5 X ocular lens with a 10 X 10 mm ocular grid were used. By turning the fine focus on the microscope 10 um at a time, we counted all leukocytes in the grid in the 10-um, 20-um, 30-um, 40-um (up to 100-um) planes from the top of the Millipore filter. (The filter is about 110-um thick.) Only PMN were present, as the rare macrophage in the preparation did not migrate into the 3-um pore filter.

The chemotaxis index was calculated by multiplying the number of cells at each plane by one-tenth the depth of the plane in the filter and then adding the resulting units. For example, 20 cells at 10 um depth gave 20 units, 15 cells at 20 um gave 30 units, 10 cells at 30 um gave 30 units, 5 cells at 40 um gave 20 units, and 1 cell at 50 um gave 5 units. The sum of these units produced a chemotaxis index of 105. The indices from the 5-grid areas were counted and then averaged.

Each culture fluid had 6 chemotaxis assays performed on it: undiluted, 1:5 dilution, and 1:25 dilution; each in duplicate. When the chemotactic factors were in high concentration, a prozone phenomenon occurred, i.e., the undiluted culture fluids showed less chemotactic activity than did the 1:5 dilution. With some culture fluids, the 1:25 dilution had the highest activity.

N-formyl-L-methanyl-L-leucyl-L-phenylalanine (FMLP) was purchased from Sigma Chemical Co. (Cat. No. F-3506) and was used in 10^{-8} to 10^{-11} M dilutions, and zymosan-activated rabbit serum (ZARS) was used in 1:10 to 1:1000 dilutions. Both FMLP and ZARS served as positive chemotactic factor controls.

The zymosan-activated rabbit serum was prepared as follows. Zymosan (from Sigma Chemical Co., Cat. No. Z-4250) was added to fresh rabbit serum, and the suspension was incubated for 30 min at 37 C, in order to activate the complement in the serum. The activated complement released the chemotactic peptide, C5a. Then, the complement was inactivated by heating the preparation at 56 C for 30 min; the zymosan particles were removed by centrifugation; and the supernatant fluid containing the C5a was aliquotted in 0.1-ml amounts, which were kept frozen at -70 C until used.

PMN and MN preparations for the Boyden chambers. Glycogen-induced PMN and MN exudate cells were obtained from the peritoneal cavities of rabbits and quantitated as described in reference 18. With both cell types, 200,000 cells were added to the top half of the Boyden chamber. The chemotactic response of these exudate cells varied from rabbit to rabbit. Therefore, only comparative results were possible.

Cleaning chemotaxis chambers. The chambers were placed in 1% Linbro 7X-O-Matic Cleaning Solution (Flow Laboratories, Inc., 7655 Old Springhouse Rd., McLean, VA 22102, Cat. No. 76-674-94), in hot tap water (about 50 C) for 1 or 2 hr, rinsed several times in hot tap water, left 1 to 3 days in deionized water (changing it 2 or 3 times a day), rinsed individually in warm tap water with a jet stream from a large pipette, rinsed again several times in deionized water, and dried in air.

EXPERIMENTAL RESULTS AND COMMENTS

Dose-response curve: Number of paranuclear vacuoles vs concentration of SM applied to human skin explants

Full-thickness skin explants (1.0 cm^2) from a breast reduction on a white, 40-yr-old female were exposed to 10 ul of 0, 0.03, 0.1, 0.3, and 1.0% SM in methylene chloride. The explants were organ-cultured for 24 hr at 36 C, fixed, embedded in glycol methacrylate, sectioned, and stained with Giemsa. The epidermal cells with paranuclear vacuoles were counted as described in the Methods section. Within this SM dosage range, there was a straight-line relationship between the dose of SM and the number of vacuolated cells (Figure 2).

Skin samples from people of different ages obtained from different areas of the body did not produce the same number of vacuoles when exposed to 0.2% SM (an intermediate SM concentration) (Table 1). We did not have enough donors in our series to identify the cause of such variation, but because of this variation, we recommend that aliquots of skin from the same human source be used for testing the comparative toxicity of a series of compounds.

Vacuole types

Paranuclear vacuoles, when studied by transmission electron microscopy (Figures 3 and 4), proved to be of two types: the "storage-type" and the "toxicant-type". The storage-type resulted when human skin was stored in the refrigerator for several days (Table 1). The toxicant-type was produced following the topical application of SM or other toxicants (6,15) or when the explants were incubated in solutions containing a toxicant (see below).

Storage-type vacuoles usually had a perinuclear location, i.e., the vacuoles partly (or fully) surrounded the nucleus. The nucleus usually projected into the vacuolar space (Figures 1 and 3). In contrast, toxicant-type vacuoles usually were found on one side of the nucleus, and the nucleus itself was usually indented by the vacuole (Figures 1 and 4).

These characteristics enabled us to identify most (but certainly not all) of the two types by light microscopy, using a 40 X objective lens (Figure 1). They also enabled us to assemble Table 1, which clearly shows that the application of SM increased the number of toxicant-type vacuoles, but did not appreciably increase the number of storage-type vacuoles.

With light microscopy, however, there was some loss of accuracy. Toxicant-type vacuoles (identified electron-microscopically by the presence of a vacuolar membrane) did not always indent the nucleus. Also, storage-type vacuoles (identified electron-microscopically by a loss of chromatin in the adjacent nucleus) could (with light microscopy) give the appearance of indenting the nucleus if the chromatin loss was mainly on one side of that nucleus.

The storage-type vacuole had no true membrane, but was merely a space in the cytoplasm, probably due to autolytic phenomena (Figure 3). The nuclear chromatin was more evenly dispersed and often appeared partly digested. Cytoplasmic organelles, such as mitochondria and endoplasmic reticulum, were fragmented. Because of the absence of a vacuolar membrane, the storage-type vacuole should not be considered a true vacuole. Nevertheless, we call them vacuoles in this report in order to make our presentation less cumbersome.

In contrast, the toxicant-type vacuole was membrane-bound (Figure 4). Such vacuolated cells usually showed no evidence of autolysis in explants incubated for 24 hr at 36 C. The toxicant-type vacuoles seemed to form while the cell still appeared essentially normal, i.e., the mitochondria and endoplasmic reticulum were still intact and not dilated.

The characteristics that distinguished the two types of vacuoles were time-dependent. The toxicant-type vacuoles developed in culture during the first 24 hr after the application of SM or other toxicants (6). By 48 hr following the application of SM, autolytic phenomena often occurred. Specifically, the vacuolar membrane became fragmented or absent, the nuclear chromatin usually became less dense, and the mitochondria and endoplasmic reticulum became abnormal in appearance.

Storage at 4 C alone can also produce (membrane-bound) toxicant-type vacuoles in some cells. Perhaps, the keratinocytes developing such vacuoles were dying at a faster rate than the others in the explant.

Effect of SM, storage, and incubation at 36 C on the two types of vacuoles

After the topical application of 0.2% SM, followed by 24 hr at 36 C, the number of toxicant-type vacuoles evaluated by light microscopy was markedly increased, but the number of storage-type vacuoles was not appreciably changed (Table 1). Since the application of the toxicant usually did not produce an increase in the number of storage-type vacuoles (which was usually low), counting all types of vacuoles in the tissue sections provided a satisfactory representation of sulfur mustard toxicity. Counting all the vacuoles also reduces the counting time and makes such counting easier to automate with a computerized image analyzer.

When studied with light microscopy, the number of toxicant-type vacuoles that formed during storage at 4 C was found to be quite variable. In fact, some of the stored skin specimens contained hardly any of these vacuoles. In contrast to skin explants stored at 4 C, explants incubated at 36 C for 3 to 4 days frequently contained toxicant-type vacuoles. Thus, toxicant-type vacuoles were produced without toxicants when the rate of epidermal cell death was hastened by the warmer temperature.

Effect of storage of human skin explants for 1 to 15 days at 4 C on the number of paranuclear vacuoles produced by SM

Human skin explants from various sources were stored in the refrigerator at 4 C for 1 to 15 days in covered Petri dishes containing our standard supplemented RPMI 1640 culture medium. Before storing, the skin was trimmed of subcutaneous fat and then cut into several pieces that fit into a standard 10-cm Petri dish. Sufficient culture medium (about 10 ml) was added so that the underside of the explants was covered, but their surface was exposed to air. The medium was changed on the days listed in Table 1, i.e., when aliquots of the skin were removed for exposure to SM.

On those days, two full-thickness explants, each 1.0 cm^2 in size, were prepared, exposed topically to 0.2% SM, and cultured (along with unexposed control explants) at 36 C for 24 hr in fresh medium. Then, two tissue sections from each explant were prepared, and the paranuclear vacuoles were counted in each section.

SM-exposed explants. Storage of human skin at 4 C from 1 to about 7 days had no consistent effect on the total number of vacuoles that were produced by a subsequent exposure to 0.2% SM and incubation for 24 hr at 36 C (Table 1): With some skin samples, such storage doubled the SM-vacuole count; with others, such storage had little effect on the count.

After about 7 days in storage, the skin specimens could no longer be used reliably to test the toxicant effect of SM. Frequently, 0.2% SM increased the vacuole count, as expected; but, sometimes, SM had little effect on the count. In the latter case, most of the epidermal cells were probably dead.

These results indicate that stored human skin specimens can be used for about a week to evaluate the toxicity of topically applied substances and the effectiveness of protective ointments and decontaminating procedures, as long as the appropriate controls are included.

Controls. Storage of human skin at 4 C from 1 to about 7 days, followed by an incubation at 36 C for 24 hr (without the application of SM), had little or no effect on the total number of vacuoles in the epidermis (Table 1). The vacuoles that did form were mainly of the storage type (Table 1).

After a week at 4 C, the total number of vacuoles increased (Table 1). This increase was probably part of the dying process.

Viability of the explants

In glycol methacrylate-embedded tissue sections, epidermal cells in skin stored over a week often appeared unhealthy when examined microscopically. Their nuclei and cytoplasm were more homogeneous and their cell boundaries were indistinct. Nevertheless, in explants from some sources, 0.2% SM caused an increase (over controls) in the number of paranuclear vacuoles (Table 1). In these explants, the increased numbers of vacuoles were probably produced by keratinocytes that were still viable when the SM was applied.

The source of the skin may affect the number of vacuoles produced in response to toxicants, but no definite pattern relating the number of toxicant-type vacuoles with source of skin was discernible. The trends suggested by the series presented in Table 1 were not reproducible when the series was extended with specimens from nine additional patients. Similarly, we were unable to relate longevity in storage with any particular source of skin. Therefore, aliquots of the same skin should be used as controls in any toxicity study.

Effect of 4- and 24-hour incubation at 36 C on the number of paranuclear vacuoles in stored human skin explants

Unexposed explants. These explants were stored in the refrigerator at 4 C for 0 to 15 days. Then, they were cut into 1.0-cm^2 pieces and incubated in RPMI 1640 medium in 5% CO_2—95% O_2 for either 4 or 24 hr. After tissue sections were made, the paranuclear vacuole counts in each explant were compared to those found in non-incubated control explants (Figure 5).

With explants that were 0 to 3 days in storage, incubation at 36 C increased the number of paranuclear vacuoles (of both types) (Figure 5). Evidently, with healthy cells, incubation accelerates the vacuolization process. With explants

that were 4 to 15 days in storage, incubation at 36 C decreased the number of paranuclear vacuoles (of both types) (Figure 5 and Table 2). The best interpretation of these results seems to be that both types of vacuolated cells died, and that during autolysis the vacuoles were altered beyond recognition.

In order to support this interpretation, rough counts were made on the epidermal cells in skin samples #2 and #3 of Table 1. A 4-hr incubation at 36 C did not appreciably affect the total number of epidermal cells (Table 2), but did substantially increase the number showing autolyzed and pyknotic nuclei (Table 2).

SM-exposed explants. Human skin explants (stored 0, 5 and 9 days) were pre-incubated (in duplicate) for 4 hr at 36 C, exposed to 0.2% SM (10 ul), and incubated for 24 additional hr. Pre-incubation of human skin explants has no consistent effects on the number of paranuclear vacuoles produced by a subsequent application of sulfur mustard (Table 3).

Azide and cycloheximide

Cell respiration is inhibited by azide, and protein synthesis is inhibited by cycloheximide. In order to determine the importance of these metabolic activities on vacuole formation, we incubated fresh human skin explants for 24 hr at 36 C in culture medium, to which sodium azide (100 ug/ml) or cycloheximide (5 ug/ml) or nothing had been added. Both inhibitors had no significant effect on the number of vacuoles produced by 1.0% SM (Table 4). The sodium azide (without SM) greatly increased the number of toxicant-type vacuoles, compared to controls, but the cycloheximide (without SM) did not do so (Table 4).

From these findings, we conclude impairment of cell respiration produces toxicant-type vacuoles, probably by initiating events that lead to the proteolytic degradation of cell cytoskeletal proteins (see Discussion). Protein synthesis is evidently not required for such vacuole production.

^{14}C-leucine incorporation test for protein synthesis

This biochemical test can be used to confirm the results of the paranuclear vacuolization test, since toxicants interfere with the incorporation of ^{14}C-leucine into the explant's proteins (6). Fresh full-thickness human skin explants, exposed to various concentrations of SM, were incubated for 24 hr with ^{14}C-leucine, along with control skin explants exposed only to the methylene chloride diluent. An inverse relationship was found between the concentration of sulfur mustard and the amount of ^{14}C-leucine incorporation by human skin explants (Figure 6). The effective concentrations of SM were the same as those producing paranuclear vacuoles in the epidermis (compare Figures 2 and 6). With rabbit skin, autoradiography showed that epidermal cells incorporated most of the ^{14}C-leucine (6).

The ^{14}C-leucine incorporation test is more cumbersome than the paranuclear vacuolization test, because it involves homogenizing the collagenous skin explants, removing the unincorporated ^{14}C-leucine with repeated washing in trichloroacetic acid, and solubilizing the proteins for scintillation counting. Both tests, however, should be equally satisfactory for testing the toxicity of chemicals applied topically to full-thickness skin explants.

Histamine release

Mast cells are plentiful in human skin. In a 1.0-cm (1 to 2-um-thick) tissue section of the seven human skin specimens studied in Table 1, we found about 100 mast cells. These cells are known to discharge histamine upon skin irritation (7-9). Therefore, it was not surprising that SM-exposed full-thickness human skin explants released more histamine into the culture fluids than did the $MeCl_2$-exposed explants (Table 5).

During 17 to 20 hr of incubation at 36 C, the difference between the SM and control groups was statistically highly significant. During 4 hr of incubation, the amount of histamine released also tended to be greater in the SM group, but this difference was not statistically significant. Either the histamine released during 4 hr had not equilibrated with the culture fluids, or there was continuous release of histamine during most of the 17- to 20-hr incubation period.

$MeCl_2$-treated and untreated explants released similar amounts of histamine in the one experiment in which we compared the two. Since $MeCl_2$ rapidly evaporates from the skin, it apparently has little or no effect on it.

Mast cell degranulation produced in vitro in human skin by the topical application of SM

In Giemsa-stained glycol-methacrylate-embedded tissue sections (1 to 2-um-thick), we counted the number of mast cells and graded the amount of degranulation 0 to ++++: 0 had no observable degranulation; +, ++, and +++ were 25%, 50%, and 75% degranulated, respectively. No ++++ degranulation was seen. A ++++ cell would be almost completely degranulated but would still contain sufficient purple granules for identification.

The degranulation of mast cells was definitely increased by the application of 1.0% SM, a finding which was statistically highly significant (Table 5). These results imply that the increased histamine found in the culture fluids from SM-treated explants was of mast cell origin.

Eicosanoids release

The topical application of 0.2% and 1.0% SM to full-thickness human skin specimens increased the release of PGE_2 into the culture fluids (Table 6). There is considerable variation in the SM effect on PGE_2 release, but our results clearly indicate that PGE_2 is an early mediator of inflammation.

Attempts to obtain ^{14}C-labeled eicosanoids (e.g., leukotriene B_4) by incubating full-thickness human skin explants with ^{14}C-arachidonic acid were unsuccessful (55a). Neither the SM-exposed nor the $MeCl_2$-exposed explants released any detectable ^{14}C-eicosanoids when the culture fluids were analyzed by high-performance liquid chromatography (55a). Both types of eicosanoid assay were performed in the laboratory of Dr. E.W. Spannhake in our department.

Lysosomal enzymes, lactic dehydrogenase (LDH), and protein release

One-square centimeter full-thickness human skin explants were exposed in vitro to 1.0% SM and cultured for 20 to 24 hr. The culture fluids were collected, cleared by centrifugation, and frozen at -70 C until they were assayed for acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, LDH, and total protein.

The activities of these enzymes (and the total protein) were similar in culture fluids from the SM- (or HN2-)exposed explants and those from the $MeCl_2$ controls (Table 7). Acid phosphatase and β-galactosidase activities were low or absent.

Triplicate human skin explants from one individual were also cultured for 3 days in order to determine whether SM hastened the release of enzymes associated with the slow cell death occurring in organ culture. The culture fluids were replaced daily and frozen until assayed. Again, little or no acid phosphatase was released from either the SM-exposed or control explants (Table 8).

Since the explants topically exposed to SM contained injured and dying epidermal cells (14), their culture fluids should have contained increased amounts of autolytic enzymes. Evidently, the changes in the amount of these enzymes released by the epidermis was too small to be detected in fluids containing similar enzymes released by dermal cells receiving little or no SM exposure.

Histochemical studies of SM-exposed and control full-thickness human skin explants

These skin explants were cultured for 4 or 18 hr after exposure to 1% SM or the methylene chloride diluent. Frozen sections were made and were stained histochemically for acid phosphatase (AP), succinic dehydrogenase (SD), and glucose-6-phosphate dehydrogenase (G6PD).

Sometimes, 1.0% SM caused increases in these enzymes in the epidermal cells of the explants, but these results were not consistently reproducible (Table 9).

Comment: Histochemical analysis can often detect changes in tissues undetectable by biochemical analysis. In our experiments, changes in enzymes in the epidermis can be visualized histochemically, whereas in the organ-culture fluids, such epidermal enzymes would form too small a component to be detectable. Unfortunately, even though the three enzymes (AP, SD, and G6PD) were readily detectable histochemically in the epidermis, no consistent effect of 1.0% SM on their activities was apparent.

Trypsin-like and chymotrypsin-like protease release

Full-thickness human skin explants, exposed in vitro to sulfur mustard, or nitrogen mustard (HN2), along with control explants, were cultured for 20 hr. The culture fluids were collected daily and frozen until assayed for trypsin-like and chymotrypsin-like proteases with the synthetic peptide substrates, L-leucyl-glycyl-L-arginyl-amino-fluorocoumarin (LGA-AFC) (36-39) and N-benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN), respectively (39-41). The levels of these proteases in the mustard group of culture fluids were not statistically different from those in the control group (Table 10).

Some of the culture fluids from each group were assayed with added plasminogen. Exogenous plasminogen helps identify the plasminogen activator (PA) in the explant culture fluids, because the additional active plasmin formed (from the inactive plasminogen) will hydrolyze LGA-AFC. Some of the culture fluids from each group were assayed with and without the plasmin inhibitor aprotinin. Aprotinin does not inhibit PA, but does inhibit plasmin (56). Finally, some of the culture fluids from each group were assayed with both added plasminogen and added aprotinin.

With every combination, the culture fluids from both SM-treated and control explants showed the same hydrolysis of LGA-AFC as the $MeCl_2$-treated controls (Table 11). About 12% of the trypsin-like activity in the culture fluids was due to the plasminogen-activator type of enzyme, and 88% of this activity was due to plasmin and other aprotinin-inhibitable proteases (Table 10).

Evidently, LGA-AFC cannot be used to detect the increase in PA activity caused by SM in human skin explants (see next section). Apparently, so many other enzymes hydrolyze this substrate that PA activity is eclipsed even in the presence of exogenous plasminogen, the PA specific substrate, and in the presence of aprotinin, which inhibits many proteases (but not PA).

Plasminogen activator (PA) release

Higher PA activity was present in culture fluids from human skin explants exposed to SM and HN2 than in culture fluids of similar explants treated with the diluent, $MeCl_2$ (Table 12). PA was measured with the ^{125}I-fibrin plate assay in the presence and absence of added plasminogen. After plasminogen was added, the increase in ^{125}I-peptide release is considered to be specific for plasminogen activators (43).

These results suggest that SM and HN2 cause the epidermal cells of the human skin explant to secrete (or release) PA. Epidermal cells are known to produce PA (57,58). Normally, the urokinase-type predominates, but in inflammatory skin conditions, keratinocytes produce much tissue-type PA (57). Other cells in the mustard lesions may also contribute to the PA that we measured, e.g., fibroblasts (43,56), macrophages (59,60), and PMN (61) and endothelial cells (62). Therefore, the mustard-injured keratinocytes may not be the only PA source.

Other factors to be considered are the following: (a) Culture fluids not containing skin explants sometimes showed greater release of ^{125}I-peptides when incubated 20 hr than those containing the explants. In other words, inhibitors of fibrin digestion were present in human skin that entered the culture fluids during the 20-hr incubation time. Thus, some of the differences between the mustard and control culture fluids might be due to differences in inhibitor content. (b) The tissue explants were not the only source of proteases. Evidently, the fibrin in the plate contained some, and the plasminogen also contained some (see the controls listed in the footnotes of Table 12). (c) Finally, many types of proteases can activate plasminogen.

Hydroxyproline release

Hydroxyproline is almost unique to collagen: other proteins contain this amino acid (46). The release of OH-proline-containing peptides into the culture fluids can, therefore, be used as a measure of collagen within the organ-cultured explants.

Collagenase may be one of the locally produced mediators of inflammation. In organ culture, intact SM lesions (produced in vivo in rabbits) released into the culture fluids more collagenase and also more OH-proline than did normal skin (63). In these experiments, both collagenase and OH-proline were higher in second- and third-day culture fluids than in first-day culture fluids (63). Since the culture fluids were changed daily, the concentration of serum protease inhibitors was less in second- and third-day culture fluids than in first-day fluids.

We, therefore, cultured full-thickness human skin explants overnight, exposed them topically to SM, and then cultured them for 3 additional days. The culture fluids were changed daily, but only the fluids from the final day were assayed for hydroxyproline (after they were cleared by centrifugation).

Unexpectedly, 1.0% SM decreased the release of OH-proline, whereas 0.2% SM sometimes increased its release (Table 13). Low concentrations of SM frequently enhanced the separation of the epidermis from the dermis, i.e., produced blisters (Table 13).

Deoxyribonuclease (DNase) and ribonuclease (RNase) release

SM is known to injure the DNA of epidermal cells (65,66). When such injury is irreversible, the DNA is hydrolyzed by DNases. Since the release of DNase into the culture fluids of SM-exposed human skin could prove to be a sensitive indicator of epidermal cell damage, we assayed these fluids for this enzyme.

Unfortunately, SM had no consistent effect on the release of DNase into the culture fluids nourishing the explants (Table 14). Similar results were found in the few such fluids assayed for RNase (also Table 14). Therefore, the assay of DNAse and RNAse does not seem to be a promising method for assessing in vitro SM injury to human skin.

In culture, more DNase was released from control rabbit skin explants than from control human skin explants (Table 14), suggesting that species differences exist. However, the topical application of nitrogen mustard to the rabbit explants still had no significant effect on the amount of DNase released (Table 14).

Angiotensin-converting enzyme (ACE)

ACE (reviewed in 48,67-69) is an ectoenzyme bound to the luminal surface of vascular endothelial cells. Upon damage, these cells release ACE into the bloodstream. It is also present in activated macrophages. Since SM damages the microvasculature of the skin, as evidenced by the resulting edematous response (14), we assayed the ACE in culture fluids of full-thickness human skin explants exposed to SM.

With o-amino-benzoylglycyl-p-nitro-L-phenylalanyl-L-proline as the substrate, human skin explants exposed in vitro to SM (or HN_2) released into the culture fluids the same (or less) ACE activity that control explants released (Table 15). A small confirmatory experiment with hippuryl-L-histidyl-L-leucine as the substrate (71), produced similar results.

Culture fluids from rabbit 1-day SM lesions showed 3 to 4 times the activity of culture fluids from explants of normal skin or 6-day SM lesions (Table 16). The ACE activity is influenced by the extravasated serum concentration in the culture fluids (Table 16).

Serum and plasma (from two rabbits) had identical ACE activities and protein concentrations. One unit of ACE activity was found in a 1:40 dilution of serum when 100 ul was assayed. Since the average protein concentration of undiluted sera was 71 mg/ml, the sera contained 5.6 units of ACE activity per mg of protein. Human serum was similar (49,50).

Summary and comment. Vascular endothelial injury produced in vitro by SM does not increase the ACE activity of human skin explants in a detectable fashion. Much of the ACE activity in these explants seems to be from the serum that these explants contain. Normal human skin contains appreciable quantities of serum. In fact, 80% of the serum albumin in the body is normally extravascular, with skin and muscle containing most of it (70).

The human skin explants have no active blood circulation. Therefore, the vascular endothelial cells in the $MeCl_2$-exposed controls may have died as rapidly as they die in the SM-exposed explants.

Interleukin 1 (IL-1) released by full-thickness human (and rabbit) skin explants exposed to SM

Interleukin 1 is a growth factor, produced by a variety of cells, including epidermal cells, for which it was originally called epidermal thymocyte activating factor (ETAF) (13). IL-1 (72) (or the IL-8 induced by it (73,74)) is chemotactic for PMN (72). We therefore assayed for IL-1 the 3-, 20-, and 48-hr culture fluids from both SM-exposed and $MeCl_2$ control human skin explants. The groups showed similar amounts of IL-1 activity (Table 17).

Since SM might stimulate macrophages to secrete IL-1, full-thickness rabbit skin explants were exposed in vitro to 1.0% SM and organ-cultured, along with control explants, in the presence and absence of autologous glycogen-induced peritoneal exudate macrophages, with and without the addition of autologous serum (Table 18). For unknown reasons, the IL-1 activity in the various culture fluids was quite variable, and no consistent effects of SM, macrophages, or serum were found. [These IL-1 assays were performed in the laboratory of Drs. Gail S. Habicht and Gregory Beck (51).]

Chemotactic factors for granulocytes and macrophages

Culture fluids from 1.0-cm^2 full-thickness human skin explants were exposed topically to 1.0% sulfur mustard (SM) (or 5.0% nitrogen mustard (HN_2). The culture fluids were collected at 3 to 4 hr and at 20 hr. They were centrifuged to remove the occasional cell and then stored at -70 C until they were assayed for chemotaxins. These assays were performed in Boyden chambers with Millipore filters for PMN chemotaxins (52,53,55) and with Nuclepore filters for macrophage chemotaxins (54). Rabbit glycogen-induced PMN and macrophage peritoneal exudate cells (18) were used as the responding cells. Since exudate cells from different rabbits vary in their response to chemotactic agents, the chemotactic activity of culture fluids from SM-exposed explants is reported as a percentage of that of $MeCl_2$ controls.

The full-thickness human skin explants, exposed topically to SM (or HN2), released smaller (not greater) amounts of chemotactic factors (for both granulocytes and macrophages) than did normal skin explants exposed only to the $MeCl_2$ diluent (Table 19). This finding was unexpected, because granulocytes and macrophages infiltrate SM lesions produced in vivo (14,18). Evidently, epidermal cells exposed to SM produce chemotactic factors early, while the cells are still alive. These factors are then destroyed or inactivated.

Complement components.

The C3a fragment from C3 cleavage is phlogistic, but not chemotactic, and the C5a fragment from C5 cleavage is both phlogistic and chemotactic. Our 3- and 17-hour culture fluids from SM-exposed and $MeCl_2$-exposed full-thickness human skin explants were assayed at the Scripps Research Institute in La Jolla, California, by Dr. Tony E. Hugli, Dr. Richard G. DiScipio, and Janet E. Wagner. C3a was assayed by a radioimmunoassay technique (75), and C5a was assayed by a chemotaxis equivalent technique (75). No statistical differences in the C3a content of the SM or $MeCl_2$ culture fluids were found, but the pooled SM culture fluids showed less C5a (chemotaxis equivalent) than did their $MeCl_2$ control (Table 20). These results were consistent with the chemotaxin assays presented in Table 19.

DISCUSSION AND CONCLUSIONS

The organ-culture system

In vitro experiments on organ-cultured full-thickness human skin explants have two major advantages over in vivo experiments: (a) Toxicants can be evaluated on human material without exposing volunteers, and (b) the initiators of the inflammatory process can be evaluated without the extravasation of serum and the infiltration of leukocytes that occur in vivo.

The disadvantages of organ culture are that its use is limited to 3 or 4 days (15) and that the circulatory and lymphatic systems are non-functional. Also, human skin is not always available from surgical procedures or recent autopsies.

General comments on the in vitro toxicity test

These studies were undertaken to develop a practical in vitro test for the toxicity of chemicals applied topically to human skin. Full-thickness human skin explants were used. Such explants proved to be remarkably hardy. In the appropriate medium, they remained viable in the refrigerator for about a week. In culture at 36 C, the explants almost always survived well for 1 day, and frequently survived for 3 or 4 days. Such hardiness is not surprising when one considers the range of temperature and variety of irritants to which human skin is frequently exposed in vivo. That human epidermal cells and hair follicle cells remain active for hours after the death of their host is further proof of such hardiness.

Full-thickness human skin explants retain their protective keratinized layer. Therefore, toxicants can be applied to their upper (dry) surface in solvents that would be injurious to cells that are not protected by a keratinized layer. Thus, except for the absence of circulating blood, these explants resemble intact skin remaining on the host.

Nature of storage-type vacuoles

The exact nature of the paranuclear vacuoles remains elusive. The storage-type seemed to be associated with gradual cell autolysis and seemed to involve solubilization of perinuclear cytoplasmic components without forming a vacuolar membrane. The storage-type vacuoles were usually associated with dispersed and sometimes partly absent nuclear chromatin. Their number was not increased appreciably following the topical application of the toxicants that we studied (6,15).

Nature of toxicant-type vacuoles

In contrast, the number of toxicant-type vacuoles was increased by the application of toxicants. This type of vacuole does not seem to have been described in the literature [except by the group at the U.S. Army Medical Research Institute of Chemical Defense (66,76-78) and ourselves (6,15)], and it does not seem to be mentioned in any current classification of cell death (79,80). A toxicant-type vacuole seems to form *in vivo* (81, and KG Moore & AM Dannenberg, unpublished observations) and *in vitro* (6,82) when a viable keratinocyte is injured and does not immediately die.

Toxicant-type vacuoles are not specific for SM injury. When added to the culture medium, sodium azide (Table 3) or calcium ionophores (C. Hirshman, unpublished experiments) produced such vacuoles in full-thickness human skin explants. Toxic doses of dinitrochlorobenzene (DNCB) or oxazolone (applied topically to full-thickness guinea pig skin explants) also produced such vacuoles (82). Sensitization of guinea pigs to DNCB or oxazolone greatly increased the number of vacuoles produced by a subsequent application of slightly toxic doses of the specific chemical (82).

The sequence leading to toxicant-type vacuole formation *in vitro* seems to occur while much of the cell's cytoplasm is still functional and intact (Figure 4), but alterations in cytoskeletal proteins probably contribute to the vacuole formation (see below). Toxicant-type vacuoles should not form when injurious agents instantly kill all functional parts of the cell, as with heat and other protein denaturants. Also, relatively few toxicant-type vacuoles formed when the epidermis died over a period of many days (during storage in the refrigerator). Thus, the rate of cell death influences the number of toxicant-type vacuoles that do form. Incubation of the explants at 36 C for 3 or 4 days often produced the toxicant-type vacuole in many of the epidermal cells (unpublished observations)--possibly because cell death occurred more rapidly than at 4 C, and possibly because the lipid membranes were more fluid at 36 C than at 4 C.

Vacuoles of the size and shape presented herein are probably characteristic of epidermal cells left in situ. The vacuoles described in liver cells (83-85) did not usually indent the nucleus to the same extent as did those produced in epidermal cells. In free cells, blebs rather than vacuoles are more commonly observed (86-89).

Origin of toxicant-type vacuoles

The membrane of the toxicant-type vacuole does not seem to originate from the nuclear envelope. Swelling of the nuclear envelope occurred, but these dilatations were small and never became as large as did many of the toxicant-type vacuoles. Toxicant-type vacuoles are probably *not* dilated endoplasmic reticulum (ER), since the ER appeared entirely normal in some of the vacuolated

cells. Epidermal toxicant-type vacuoles probably form by invagination of the plasma membrane, similar to the vacuoles in liver cells of animals made anoxic (83,84) or killed by electic shock (85).

The biochemical mechanisms involved in the formation of toxicant-type vacuoles are probably those described by Trump and co-workers (88,89). Specifically, the following sequence of events probably occurs: Sulfur mustard, azide, and other toxicants interfere with cell respiration, thereby reducing the cell's ATP energy stores. [SM alkylates DNA. Such DNA injury activates poly(ADP-ribose) synthetase (90), which depletes the cell's NAD, a co-enzyme necessary for cell respiration (91).] The decrease in ATP energy stores reduces the cell's ability to maintain Ca^{++} homeostasis. Therefore, cytosolic Ca^{++} increases, and Ca^{++}-dependent neutral proteinases are activated (86,87). These proteinases degrade cytoskeletal proteins and/or membrane-integral proteins (86,87), which, in intact epidermis, makes conditions favorable for plasma membrane invagination and vacuole formation.

Mediators that initiate the inflammatory response in human skin

These initial mediators undoubtedly arise from cells stimulated or injured by the irritant. Histamine, prostaglandin E_2, and plasminogen activator were identified in our present studies.

Histamine most likely is released from the mast cells in the explants, and these cells were more degranulated in SM-treated than in the control explants. Mast cells are particularly sensitive to irritation and seem to be involved in such basic inflammatory phenomena as Sir Thomas Lewis's triple response (9).

PGE_2, one of the eicosanoids, is released when the cell's external membrane is perturbed (8). Mol has identified increases in PGE_2 and 6-keto-PGF_1(alpha) after SM exposure of human keratinocytes to 200 uM SM (personal communication). 6-keto-PGF_1 (alpha) is the stable degradation product of the proinflammatory PGI_2 (prostacyclin). Thus, along with histamine from mast cells, eicosanoids (presumably from irritated keratinocytes) may be among the major local initiators of the inflammatory process. In our experiments, culture fluids from full-thickness human skin explants exposed to SM were assayed for PGE_2. SM usually increased the levels of this eicosanoid (Table 6).

Plasminogen activator (PA) is one of the proteases known to be associated with blister formation in human skin (43,44,92,93). PA probably is produced by the keratinocytes after irritation or injury by SM. However, we cannot rule out PA production by the local fibroblasts (42,43), which may also be activated by SM (18).

Recently, many cytokine mediators of the inflammatory response have been identified and characterized (12). Their roles as initial mediators of the inflammatory response remain to be determined. The evaluation of various cytokines in the pathogenesis of SM lesions is the mission of our present U.S. Army contract. The half-lives of many of these mediators (cytokines) are short (12,96), and inhibitors (12) are often present. Thus, it is possible that many of these cytokines could initiate the inflammatory response and be undetectable in our organ-culture fluids.

For this reason, our studies provide only part of the answer to the question: What mediators initiate the inflammatory response? They represent an adequate beginning, but the full list will probably not be known for many years to come, if ever.

Figure 1. Representative paranuclear vacuoles in human skin stored for 10 days at 4 C. A typical toxicant-type vacuole indenting the nucleus (first large arrow), and a typical storage-type vacuole wrapped around the nucleus (second large arrow) are depicted in the center of the epidermis. Three small toxicant-type vacuoles (small arrows) are also present. The vacuoles in pyknotic cells (e.g., the central basal cells herein) are, routinely, not counted. Also, vacuoles smaller than those marked with small arrows are, routinely, not counted. Precise differentiation of storage-type and toxicant-type of vacuoles is not possible without electron microscopy, but indentation of the nucleus as a criterion seems to be satisfactory. Giemsa stain. X 625 (i.e., 0.625 mm = 1.0 um).

Figure 2. The effect of various concentrations of SM on the number of paranuclear vacuoles in epidermal cells of fresh full-thickness human skin explants. The 1.0-cm^2 explants received a topical application of 10 ul SM and then were cultured at 36 C for 24 hr. The vacuolated epidermal cells were counted microscopically in glycol-methacrylate-embedded tissue sections. The means (and their standard errors or range) of two to four explants are presented. These explants came from skin removed surgically for a breast reduction.

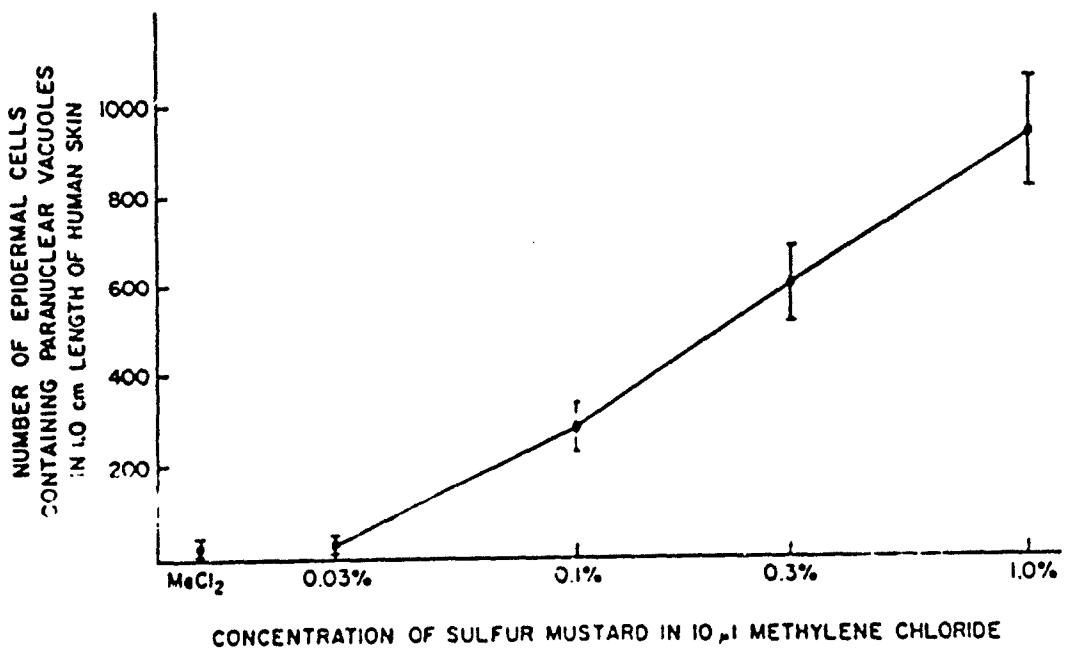

Figure 3. A degenerated cell in a full-thickness human skin explant stored at 4 C for 4 days. The empty space around the nucleus illustrates a storage-type of paranuclear vacuole (at the ultrastructural level). It is not membrane-bound, but is surrounded by fragments of cytoplasm, including swollen and fragmented mitochondria and numerous keratohyalin filaments. The nucleus is disintegrating, but the nuclear membrane is largely intact. The chromatin appears more evenly dispersed because of decreased electron density of the heterochromatin (probably caused by lysis). (X 16,500.)

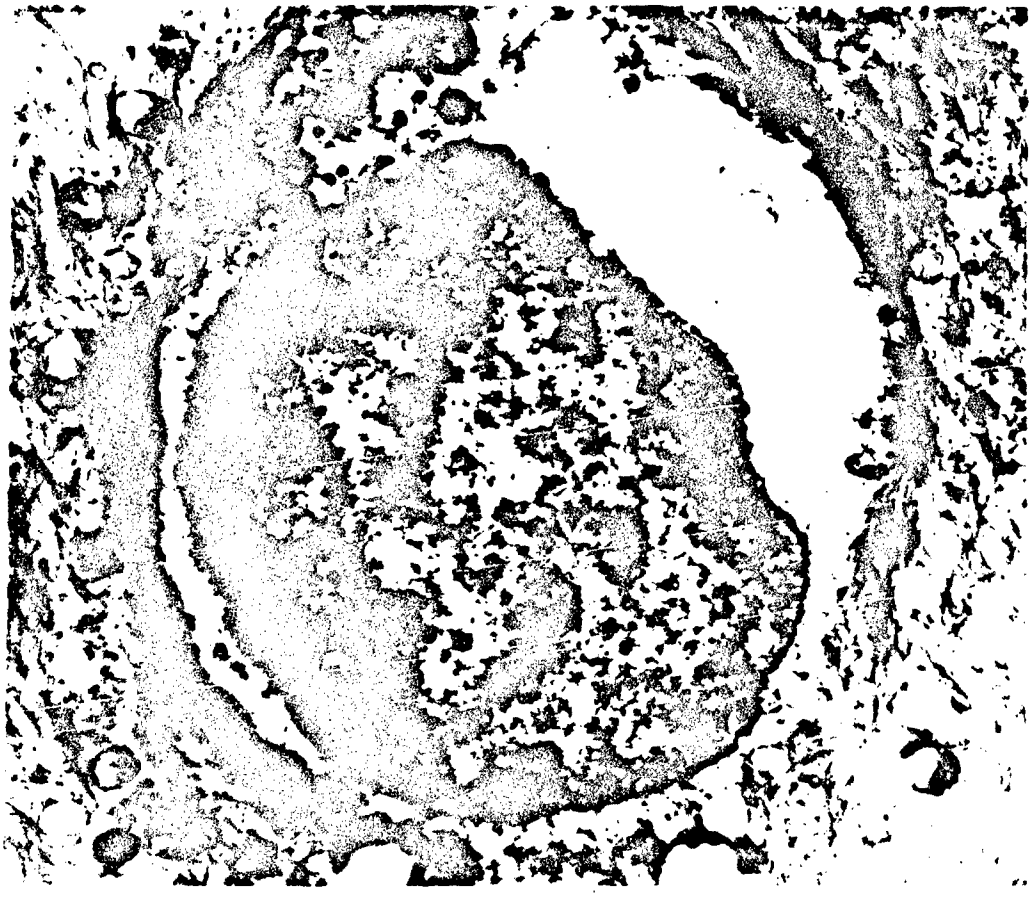

Figure 4. A cell in a full-thickness human skin explant exposed topically to 1.0% SM and cultured for 48 hr. Depicted is a typical toxicant-type paranuclear vacuole indenting one side of the nucleus. Note that this vacuole is membrane-bound, in contrast to the storage-type vacuole. The nuclear chromatin is condensed. The nuclear envelope is largely intact, but in this case a split in the envelope is present. Explants cultured for the standard 24 hr showed similar vacuoles and, usually, better preservation of cytoplasmic organelles. X 22,700.

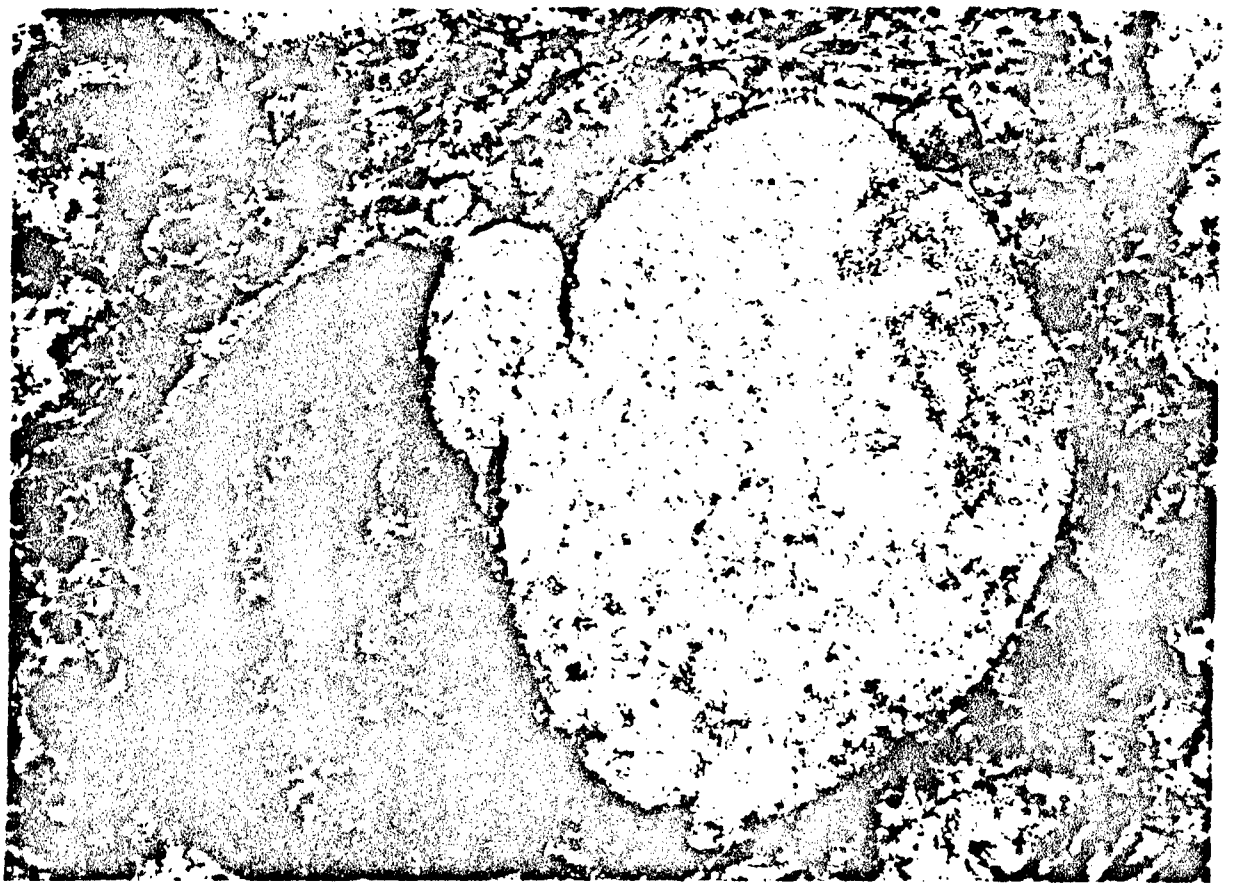

Figure 5. Effect of storage at 4 C, and incubation at 36 C, on the number of paranuclear vacuoles in full-thickness human skin explants. Skin specimens from 7 patients were stored in culture medium in the refrigerator at 4 C for the days listed in Table 1. Tissue sections were prepared, and the paranuclear vacuoles were counted. After 0 to 3 days of storage, the vacuole count ranged from 4 to 117 per cm among the 7 sources of skin. After 4 to 7 days of storage, the vacuole count ranged from 100 to 810; and after 8 to 15 days of storage, it ranged from 158 to 810. For each time period, with each patient, these "after-storage" vacuole counts are represented as 100%. The effects of 4- and 24-hr incubation at 36 C on these "after-storage" counts are depicted in this graph (with means and their standard errors). Note that the number of vacuoles increased with incubation (or remained the same) when the skin specimens were fresh, but decreased with incubation when the skin specimens had been aged at 4 C.

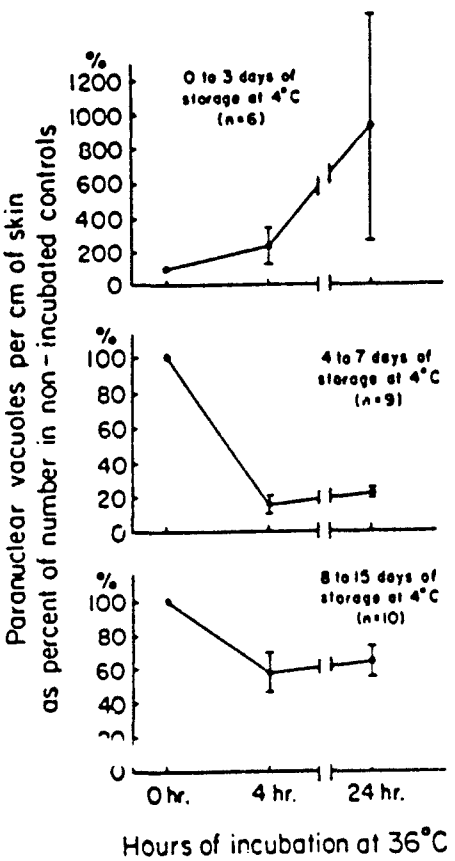

Figure 6. The effect of various concentrations of SM on protein synthesis in fresh full-thickness human skin explants, determined by the amount of ^{14}C-leucine incorporation in a 24-hr organ culture. A decrease in protein synthesis, i.e., leucine incorporation, is a measure of toxicity to the explant. o represents explants from one patient; □ represents explants from the other patient. The line connects the means. The lowest concentration of SM with distinct effects on every explant was 0.1%.

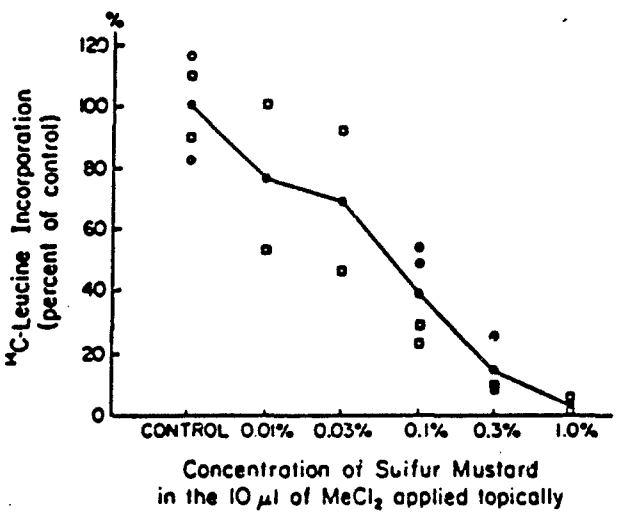

Table 1: Effect of storage of full-thickness human skin explants at 4 C on the number of paranuclear vacuoles in SM-exposed and control groups after incubation at 36 C for 24 hr

Skin sample no. and type of skin	Days in storage at 4 C	Number of paranuclear vacuoles in a central 1.0-cm tissue section across the explant after 24-hr incubation at 36 C					
		Controls			0.2% SM		
		storage type	toxicant type	total	storage type	toxicant type	total
#1							
Normal breast	1 day(s)	--	--	--	28	710	738
skin, black	4 "	13	2	15	28	1310	1338
female, age 30	7 "	13	10	23	28	1040	1068
#2							
Normal breast	0 day(s)	45	14	59	35	780	815
skin, white	5 "	63	28	91	41	470	511
female, age 27	9 "	28	13	41	56	910	966
#3	2 days	49	16	65	49	910	959
Normal breast	5 "	40	9	49	56	1060	1116
skin, black	8 "	15	10	25	29	1030	1059
female, age 24	12 "	87	240	327	40	1010	1050
	15 "	15	123	138	30	870	900
#4							
Normal leg	3 days	20	11	31	22	260	282
skin, white	6 "	20	10	30	20	400	420
female, age 79	10 "	26	59	85	28	220	248
#5	1 day(s)	9	4	13	27	161	188
Normal leg	4 "	24	4	28	44	340	384
skin, white	8 "	57	115	172	41	187	228
male, age 72	15 "	172	58	230	124	168	292
#6							
Normal arm	7 days	29	34	63	78	370	448
skin, white	14 "	270	176	446	240	270	510
male, age 30							
#7							
Normal breast	0 day(s)	25	22	47	46	330	376
skin, white	6 "	34	49	83	60	420	480
female, age 51	12 "	230	360	590	350	500	850

Table 1: Effect of storage of full-thickness human skin explants at 4 C on the number of paranuclear vacuoles in SM-exposed and control groups after incubation at 36 C for 24 hr (continued)

The skin was received in our laboratory 2 to 4 hr after surgery. It was kept refrigerated at 4 C until incubated for 4 or 24 hr at 36 C. Two explants were cultured for each value listed, and the paranuclear vacuoles were counted in two tissue sections prepared from each explant. The mean of these four paranuclear counts was then calculated. The standard errors of these means were usually between 5 and 15% of their respective means.

SM (0.2% in $MeCl_2$) was topically applied to the stored full-thickness skin specimens within 2 hr of their removal from the refrigerator. The controls received no topical application.

These data show that 0.2% SM increased the total number of paranuclear vacuoles, both in fresh skin specimens and in those stored at 4 C. This increase was mainly due to an increase in the number of toxicant-type vacuoles, as the number of storage-type vacuoles was not appreciably increased by SM. Additional data on the number of paranuclear vacuoles produced by 1.0% SM are attached as Table 21 (pp. 51-52) in the Addendum, but the series is still too small to make any correlation between the source of skin and the total number of vacuoles produced by SM.

Table 2: Characteristics of epidermal cells in 1.0-cm^2 full-thickness skin explants stored at 4 C for 5 days and then incubated for 4 hr at 36 C

Cell characteristics	Number of cells in an average 1.0 cm of epidermis			
	#2 5-day storage and no incubation	#2 5-day storage and 4-hr incubation	#3 5-day storage and no incubation	#3 5-day storage and 4-hr incubation
Storage-type vacuoles	83	4	110	3
Toxicant-type vacuoles	180	36	98	12
Pyknotic nuclei	125	320	165	255
Autolyzed nuclei	1500	2250	1500	2200
Degenerative changes	2600	2650	1200	1900
No change	1000	1050	2150	2350
Total cells	5488	6310	5223	6720

The number of cells with each characteristic in a 1.0-cm length of epidermis was determined in the following manner: For each figure listed, 8 ocular grid widths were chosen randomly in the epidermis of 3 tissue sections. Under a 40 X objective, the epidermal cells with discernible nuclei were counted and characterized in these grid widths. Then, the means (and their standard errors) of the 8-grid area counts were calculated and multiplied by 50 to get the total vacuole count.

Table 3: The effect of storage, incubation, and SM on storage-type and toxicant-type paranuclear vacuoles in full-thickness human skin explants

Days in storage at 4 C	Controls Incubation time at 36 C 0 hr	4 hr	24 hr	Sulfur mustard Incubation time at 36 C 24 hr	4-hr preincubation 24-hr incubation
0 day					
Storage-type vacuoles	1 ± 1	2 ± 1	50 ±12	30 ± 1	20 ± 5
Toxicant-type vacuoles	1 ± 1	1 ± 1	16 ± 4	670 ±80	300 ±30
Total vacuoles	2 ± 1	3 ± 1	66 ±16	700 ±80	320 ±40
5 days					
Storage-type vacuoles	56 ±11	4 ± 1	55 ± 4	44 ± 5	27 ± 4
Toxicant-type vacuoles	119 ±15	31 ± 4	24 ± 3	530 ±50	610 ±20
Total vacuoles	175 ±30	35 ± 3	79 ± 6	574 ±50	637 ±20
9 days					
Storage-type vacuoles	75 ± 7	14 ± 1	24 ± 3	51 ±10	46 ± 6
Toxicant-type vacuoles	60 ±24	12 ± 2	12 ± 3	820 ±50	1220 ±90
Total vacuoles	135 ±28	26 ± 3	36 ± 5	871 ±50	1266 ±90

The data presented are from a representative patient. The complete recounts on explants from four other patients were also made. In all four, our findings were similar to those presented here.

The data show that preincubation of stored skin for 4 hr at 36 C had no consistent effect on the number of paranuclear vacuoles produced by SM in organ-cultured skin explants. The data also show that the toxicant SM increases the number of toxicant-type vacuoles, but does not increase the number of storage-type vacuoles. (The control group provides a specific example of data summarized in Figure 5.)

Table 4: Effect of sodium azide and cycloheximide on the number of epidermal paranuclear vacuoles produced in full-thickness human skin explants by SM

Treatment	Topical treatment of explant	
	$MeCl_2$ (diluent control)	Sulfur Mustard (1.0% in $MeCl_2$)
Sodium azide (100 mg/ml)		
Storage-type vacuoles	69 ± 14	119 ± 22
Toxicant-type vacuoles	590 ±290	830 ±260
Total vacuoles	660 ±290	950 ±260
Cycloheximide (5 ug/ml)		
Storage-type vacuoles	69 ± 34	75 ± 10
Toxicant-type vacuoles	11 ± 2	970 ±250
Total vacuoles	80 ± 33	1050 ±250
Nothing		
Storage-type vacuoles	88 ± 59	89 ± 16
Toxicant-type vacuoles	12 ± 4	800 ±220
Total vacuoles	100 ± 63	890 ±230

Toxicant-type vacuoles were evidently produced when full-thickness human skin explants were cultured for 24 hr in the presence of sodium azide (100 ug/ml), but no such vacuoles were produced in the presence of cycloheximide (5.0 ug/ml). Neither inhibitor had much effect on the number of vacuoles produced by 1.0% sulfur mustard. These experiments were performed on either the same day that the skin was removed from the patient, or after it was stored overnight at 4 C in culture medium. The means (and their standard errors) of experiments on skin from 5 patients are listed.

Table 5: Histamine release and mast cell degranulation produced by topical application of SM or $MeCl_2$

Skin specimen number	Histamine ng/ml		Weighted number of degranulated mast cells	
	1.0% SM	$MeCl_2$ (controls)	1.0% SM	$MeCl_2$ (controls)
# 710	48	25	65*	34
# 206	45	23	60	32
# 620	43	20	54	29
# 515	41	21	70	23
# 428	30	22	53	13
# 412	26	16	46	19
# 427	24	15	61	33
Mean and its SE	37 ±4	20 ±1	58 ±3	26 ±3

The amount of histamine in the 20-hr culture fluids was listed in descending order. Then, the weighted numbers of degranulated mast cells in the same skin specimen were listed.

* The weighted number of degranulated mast cells was derived as shown in the following example: #710 had 15 +, 16 ++, and 6 +++ mast cells in the 1.0-cm tissue section: 15 X 1 added to 16 X 2 added to 6 X 3 = 65. Since "+" represents 25% degranulation, ++ 50%, and +++ 75%, this weighting seems logical.

The topical application of 1.0% SM released more histamine and degranulated more mast cells than did the topical application of $MeCl_2$ (P <0.0005 and <0.0001, respectively). However, in skin specimens from the same human source, no strict correlation was found between the weighted number of degranulated mast cells and the amount of histamine released. Probably, the 1- to 2-um tissue section does not adequately represent the total number of mast cells in the entire 1.0-cm^2 explant.

Table 6: PGE_2 in culture fluids from full-thickness human skin explants topically exposed *in vitro* to SM or $MeCl_2$

Human skin specimen	PGE_2 in $MeCl_2$ (controls) (pg/ml)		Increase (or decrease) 4 hr in culture		Increase 18 hr in culture	
	Time in culture 4 hr	18 hr	0.2% SM	1.0% SM	0.2% SM	1.0% SM
#1	440	1150	+14%	+23%	+23%	+26%
#2	470	890	+57%	+34%	+47%	+37%
#3	23	220		-91%		+109%
#4	79	520		+10%		+105%
#5	23	460		+480%		+10%

From this data, we conclude that PGE_2 is usually, but not always, increased by the topical application of SM.

Because of occasional very large values, the statistical tests based on normal distribution are not appropriate. Therefore, the nonparametric sign test, based on the binomial distribution, was used (64). Specifically, with seven control-experimental (C-E) pairs, the probability of E being greater than C seven times is 0.008 (and six times is 0.054).

Table 7: Lysosomal enzymes and lactic dehydrogenase (LDH) in culture fluids from 1.0-cm^2 human skin explants topically exposed in vitro to SM or nitrogen mustard (HN2)

Enzyme (or protein)	3-hr organ-culture fluids		20-hr organ-culture fluids	
	$MeCl_2$	1% SM or 5% HN2	$MeCl_2$	1% SM or 5% HN2
Acid phosphatase nmol NP*/ml	12.5 ± 8.2	17.4 ± 10.3	16.4 ± 10.1	6.5 ± 5.7
β-Glucuronidase nmol NP*/ml	37 ± 20	35 ± 19	85 ± 45	78 ± 45
β-Galactosidase nmol NP*/ml	0	0	0.65 ± 0.59	0
Lysozyme ug/ml	2.0 ± 0.2	2.3 ± 0.5	2.8 ± 0.3	2.9 ± 0.3
Lactic dehydrogenase mU/ml	24 ± 10	23 ± 10	37 ± 17	39 ± 14
Protein concentration mg/ml	0.20 ± 0.02	0.19 ± 0.02	0.44 ± 0.05	0.46 ± 0.09

*NP = p-nitrophenol

The explants were cultured either 3 or 20 hr after 10 ul of 1% SM or 5.0% nitrogen mustard (HN2) was applied to their surfaces. The skin from three patients was used for the 3-hr cultures, and skin from six patients was used for the 20-hr cultures. Skin explants from one third of the donors were exposed to SM, and skin explants from two thirds of the donors to HN2.

The means and their standard errors are listed. No statistically significant differences were found between explants exposed to the mustards and those exposed to the $MeCl_2$ diluent. Almost half of the explant culture fluids assayed had no detectable acid phosphatase activity and almost all of those assayed had no detectable β-galactosidase activity.

Table 8: Acid phosphatase activity in organ-culture fluids from 1.0% SM-exposed and control full-thickness human skin explants

Day culture fluid was collected	Incubation time for acid phosphatase	Culture fluids incubated without skin explants	Culture fluids from SM-exposed skin explants	Culture fluids from $MeCl_2$-exposed skin explants	Amount of culture fluid in assay
		Optical density at 410 um			
First day	30 min	0.030-0.045	0.030-0.040	0.030-0.040	0.2 ml in 3.2 ml
Second day	"	"	0.035-0.045	0.030-0.045	
Third day	"	"	0.035-0.040	0.035-0.035	
First day	20 hr	0.101-0.104	0.095-0.110	0.095-0.100	0.5 ml in 3.5 ml
Second day	"	"	0.105-0.130	0.090-0.105	
Third day	"	"	0.100-0.110	0.095-0.100	

The optical density ranges of triplicate human skin explants are presented. An optical density reading of 0.100 was equivalent to 2.7 ug of nitrophenol in 3.0 ml read in the cuvette at 410 nm.

Incubation of the culture fluid without the skin explant provides a measure of the spontaneous hydrolysis of the nitrophenyl phosphate substrate during the acid phosphatase assay procedure. This control proved that the culture fluids from the human skin explants had minimal, if any, acid phosphatase activity.

Table 9: Histochemical demonstration of acid phosphatase (AP), glucose-6-phosphate dehydrogenase (G6PD) and succinic dehydrogenase (SD) in the epidermis of full-thickness human skin specimens topically exposed to SM in vitro and organ-cultured

	4 hr in organ culture			18 hr in organ culture		
Enzyme	0.2% SM exposed	1.0% SM exposed	$MeCl_2$ exposed	0.2% SM exposed	1.0% SM exposed	$MeCl_2$ exposed
AP	++	+++ +++	+ +++	+	++++ ++	++ ++
G6PD	++	++++ +++	+++ +++	++	++++ +	++ ++
SD	+++	+++	+++	+	+	++

The average observation on duplicate (unfixed) tissue sections from each patient is listed. No consistent effects of SM on these epidermal enzymes were found.

Table 10: Trypsin-like and chymotrypsin-like proteases in culture fluids from 1.0-cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard (HN2)

Treatment of explant	Trypsin-like protease			Chymotrypsin-like protease
	LGA-AFC (fluorescence units) A	LGA-AFC + Aprotinin (2.5 ug/ml) (fluorescence units) B	Percent of protease activity remaining (B/A x 100)	(BPN) (optical density units)
1% SM or 5% HN2	41.1 ±10.5	4.5 ±0.6	11%	0.078 ±0.005
$MeCl_2$	47.5 ±10.1	6.8 ±2.0	14% (average = 12%)	0.086 ±0.016

Substrates: LGA-AFC = leucyl-glycyl-arginyl-aminofluorocoumarin;
BPN = N-benzoyl-phenylalanine- -naphthyl ester.

The full-thickness skin explants from 5 or 6 patients were exposed to 1% SM or 5% HN_2 and then were organ-cultured in triplicate for 20 hr in RPMI 1640 medium. (Only one of the 5 or 6 groups was exposed to HN2 instead of SM.) The triplicate culture fluids were pooled and assayed for protease activity with LGA-AFC and BPN. Aprotinin inhibits plasmin and trypsin, but does not inhibit plasminogen activator (56). The means and their standard errors are listed.

No statistical differences were found in the trypsin-like proteases hydrolyzing LGA-AFC or in the chymotrypsin-like proteases hydrolyzing BPN when the culture fluids from mustard-treated and those from diluent-treated explants were compared.

Table 11: The effect of added plasminogen and/or aprotinin on the trypsin-like enzymes in organ-culture fluids from full-thickness human skin explants topically exposed *in vitro* to 1.0% SM or $MeCl_2$

Substances added	Source of culture fluid	SM-exposed skin ug AFC released per 50 ul culture fluid	$MeCl_2$-exposed skin ug AFC released per 50 ul culture fluid
Plasminogen and aprotinin	A	6.5	6.4
	B	7.3	7.4
Plasminogen	A	10.3	10.2
	B	10.9	11.1
Aprotinin	A	0.18	0.25
	B	0.12	0.12
Nothing added	A	0.55	1.33
	B	0.46	0.18

The incubated mixture contained culture fluids (50 ul), 0.05 M TES buffer, pH 8.2 (800 ul), plasminogen 1.25 units in 0.9% NaCl (50 ul), and/or aprotinin 2.5 ug in 0.9% NaCl (50 ul), LGA-AFC substrate 20 mM in dimethyl formamide (50 ul), and sufficient 0.9% NaCl to make a 1.0-ml final volume. TES designates N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid. Twenty-hour culture fluids from skin explants of two patients (A and B) were assayed.

SM-exposed explants and control explants showed no consistent differences in protease activity measured with LGA-AFC (Table 10). The above table shows the same result following the addition of plasminogen, the substrate for plasminogen activator (PA), and/or the addition of aprotinin, an inhibitor of trypsin and plasmin (but not of PA).

Table 12: Plasminogen activator activity in organ culture from 1.0-cm^2 full-thickness human skin explants topically exposed *in vitro* to SM or nitrogen mustard (HN2)

Skin Sample Number and Treatment	Amount of Plasminogen Added per well (ug)	Radioactivity of ^{125}I-peptides released from ^{125}I-Fibrin by the Explant Culture Fluids in 1.5 hr at 37 C					
		Plasminogen present		Plasminogen absent		Difference	III Change due to added plasminogen in percent I/II x 100
		I cpm	percent E/C x 100[a]	II cpm	percent E/C x 100[a]	cpm	
9[b] $MeCl_2$	5	6398		6679		-281	96 %
1% SM		9108	142 %	6839	102 %	2269	133
10[b] $MeCl_2$	5	5398		8007		-2609	67
1% SM		6995	130	5780	72	1215	121
11[b] $MeCl_2$	5	7627		6981		646	109
5% HN2		8585	113	6997	100	1588	123
11[c] $MeCl_2$	1.25	1697		1399		298	121
5% HN2		3431	202	1386	99	2045	248
12[c] $MeCl_2$	1.25	3007		1725		1282	174
5% HN2		22089	735	6812	395	15277	324
13[c] $MeCl_2$	1.25	2964		1664		1303	178
5% HN2		15826	534	1697	102	14129	932
14[d] $MeCl_2$	2.5	442		423		19	104
1% SM		499	113	413	98	86	120
15[d] $MeCl_2$	2.5	463		362		101	127
1% SM		682	147	421	116	261	162
Means and their standard errors:		265% ±84% ($P = 0.004$)[e]		136% ±37 (N.S.)[e]		C = 122% ±13% E = 270% ±98% ($P = 0.004$)[e]	

Table 12: Plasminogen activator activity in organ culture from 1.0-cm^2 full-thickness human skin explants topically exposed in vitro to SM or nitrogen mustard (HN2)

(continued)

The explants were exposed to 1.0% SM, 5.0% HN2, or $MeCl_2$ diluent, and incubated in the RPMI 1640 culture medium for 20 hr at 37 C. The culture fluids were frozen until assayed for plasminogen activator (PA) by the fibrin plate method. Then, 50 ul was added to each well and the samples were incubated for 1.5 (or 3) hr.

The figures in columns I and III clearly indicate that the mustards increase the production (or release) of PA by the skin explants (P = 0.004). Plasminogen is the substrate for PA.

The variations in radioactivity released were due to fibrin plates having been made at different times and stored for different periods of time. The half-life of ^{125}I is 60 days.

[a] Experimental group (E) (SM or HN2) divided by control group (C) ($MeCl_2$) times 100.

[b] Controls: There were 3318 ^{125}I-cpm (in 200 ul) when fresh RPMI medium with plasminogen (5 ug) was added to the fibrin plates and incubated 1.5 hr at 37 C. (No controls without plasminogen were included in this group of experiments.)

[c] ^{125}I-fibrin plates containing culture fluids from these explants were incubated 3 hr instead of 1.5 hr. Controls: There were 2084 ^{125}I-cpm (in 200 ul) when fresh RPMI medium was added to the fibrin plates and incubated for 3 hr at 37 C. When plasminogen (1.25 ug) was included with this control, the counts were 4277/min.

[d] Controls: There were 1565 ^{125}I-cpm (in 200 ul) when fresh RPMI medium, without skin explants, was added to the fibrin plates and incubated for 1.5 hr at 37 C. When plasminogen (2.5 ug) was included with this control, the counts were 1971/min.

[e] Because of occasional very large values, the statistical tests based on normal distribution are not appropriate. Therefore, the nonparametric sign test, based on the binomial distribution, was used (64). Specifically, with eight control-experimental (C-E) pairs, the probability of E being greater than C eight times is 0.004 (and seven times is 0.03). N.S. = no significant difference.

Table 13: The effect of 0, 0.2, and 1.0% SM on hydroxyproline release and blistering by full-thickness human skin explants (previously cultured overnight) and then cultured an additional 3 days

Skin specimen number	$MeCl_2$		0.2% SM		1.0% SM	
	OH-proline ug/ml*	Degree of blistering	OH-proline ug/ml*	Degree of blistering	OH-proline ug/ml*	Degree of blistering
302	2.	+ to ++	11.	+++ to ++++	0.2	0 to ±
427	95.	0 to ±	98.	+++ to ++++	9.	0 to ±
620	29.	+ to ++	109.	+ to ++	24.	0 to ±
710	8.	0 to ±	9.	+++ to ++++	4.	+ to ++
724	18	0 to ±	6.	0 to ±	2.	+ to ++

The term "blistering" is used to designate separation of the epidermis from the dermis seen in tissue sections: 0 = no separation; ++++ = complete separation; and ++ = 50% separation.

No relation between source of skin, OH-proline release, and blistering was apparent.

* ug OH-proline per ml of third-day culture fluid. Each 1.0-cm^2 explant was cultured in 2.5 ml of Dulbecco's Modified Eagle Medium. This medium was changed daily.

Table 14: DNase and RNase activity in organ-culture fluids from full-thickness human (and rabbit) skin explants topically exposed *in vitro* to SM or nitrogen mustard

Enzyme	No. and type of skin	Mustard-exposed	$MeCl_2$-exposed
		change in optical density at 260 nm	change in optical density at 260 nm
DNase	6 human skins	SM 0.026 ±0.037	0.018 ±0.026
	8 rabbit skins	HN2 0.140 ±0.024	0.108 ±0.027
RNase	3 human skins	SM 335-490 (range)	175-330 (range)

The full-thickness skin explants were organ-cultured for 17 to 24 hr. Then, culture fluids were collected, centrifuged, and stored at -70 C until assayed for DNase or RNase, with a 2-hr incubation period at 36 C. There were 2 to 4 explants from each skin specimen.

In the experiments with rabbit skin, 5% nitrogen mustard (HN2) (10 ul), instead of 1% sulfur mustard (10 ul), was applied topically *in vitro* to the full-thickness skin explants, because SM could not be obtained at that time.

SM or HN2 had no consistent effect on the DNase and RNase released into the culture fluids.

Table 15: Angiotensin-converting enzyme activity in culture fluids (CFs) from 1.0-cm^2 human skin explants topically exposed in vitro to SM or nitrogen mustard

Skin sample number and treatment	Time after SM exposure at which CFs were collected	ABz-Gly released		Comments	
		Fluorescence units[a]	Percent (E/C x 100)	Sample size (ul)	Incubation time with substrate for ACE
11 Nothing	5 hr	2.10	100 %	2000	4 hr
1% SM		2.10			
14 $MeCl_2$	4 hr	0.06		50	45 min
1% SM		0			
16 $MeCl_2$	3 hr	0.01	100	50	45 min
5% $HN2$		0.01			
17 $MeCl_2$	3 hr	0.25	80	100	2 hr
5% $HN2$		0.20			
18 $MeCl_2$	3 hr	0.05		100	2 hr
5% $HN2$		0			
Mean and its standard error:			56% ±23%		
9 $MeCl_2$	20 hr	0.63	95 %	500	45 min
1% SM		0.60			
10 $MeCl_2$	20 hr	0.51	94	500	45 min
1% SM		0.49			
14 $MeCl_2$	20 hr	0.03	67	50	45 min
1% SM		0.02			
16 $MeCl_2$	20 hr	0.05	60	50	45 min
5% $HN2$		0.03			
17 $MeCl_2$	20 hr	1.40	57	100	45 min
5% $HN2$		0.80			
18 $MeCl_2$	20 hr	0.25	60	100	45 min
5% $HN2$		0.15			
Mean and its standard error:			72% ±7%		

continued

Table 15: Angiotensin-converting enzyme activity in culture fluids from 1.0-cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard

(continued)

* Fluorescent units: In our experiments, one fluorescent unit was equivalent to 1.5 nmol of ABz-Gly released from the ACE substrate, o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (49). ACE activity was the difference in the fluorescent units produced by a lesion culture fluid incubated with the substrate without EDTA and the units produced with the substrate in the presence of EDTA (which inactivates ACE).

Table 16: Angiotensin-converting enzyme activity in culture fluids from 1.0-cm^2 rabbit SM lesion explants and control skin

Source of CFs	ABz-Gly Released in 45 min by 100 ul CFs (units)[b]	Protein Concentration (mg in 100 ul)	ABz-Gly Released per mg of Protein (units)[b]	Contribution of Serum to ACE Activity in Lesions[a] (units)[b]
Normal skin	A 0.28 ±0.05	0.05 ±0.005	D 5.8 ±0.8	0.24 (86%)
1-day SM lesion	B 1.01 ±0.07	0.16 ±0.014	E 6.2 ±0.4	0.76 (75%)
6-day SM lesion	C 0.32 ±0.08	0.10 ±0.007	F 3.4 ±0.8	0.48 (150%)

The means and their standard errors are listed. Culture fluids (CFs) of lesions and of normal skin from each of five rabbits were assayed. When the matched-paired Student's t-test was used: A vs. B, $P < 0.004$; A vs. C, N.S.; B vs. C, $P < 0.004$; D vs. E, N.S.; D vs. F, $P < 0.04$; E vs. F, $P < 0.004$. N.S. = not statistically significant.

[a] We assumed that the protein in the culture fluids from the lesions (and normal skin) is 85% serum protein (94,95). One milligram of serum protein contained 5.6 units of ACE activity.

[b] In our experiments, one fluorescent unit was equivalent to 1.5 nmol of ABz-Gly released from the ACE substrate, o-aminobenzoylglycyl-p-nitro L-phenylalanyl-L-proline (49). ACE activity was the difference in the fluorescent units produced by a lesion culture fluid incubated with the substrate without EDTA and the units produced with the substrate in the presence of EDTA (which inactivates ACE) (see Materials and Methods).

Table 17: Interleukin 1 activity of organ-culture fluids from human skin topically exposed in vitro to 1.0% SM

Time in organ culture at 36 C	Human skin specimen	SM (dpm X 10^{-2})	$MeCl_2$ (dpm X 10^{-2})
3 hr	A	10.3 ±0.7	14.9 ±1.7
	B	12.9 ±2.6	9.1 ±1.2
20 hr	A	16.2 ±4.1	12.7 ±3.1
	B	9.1 ±0.6	10.0 ±1.6
48 hr	A	----	----
	B	13.2 ±1.4	13.3 ±1.0

Listed are the radioactive tritium disintegrations per min at a 1:16 dilution of (dialyzed) culture fluid (see Experimental Methods). Values above 10.0 (i.e. 1000 dpm) can be considered to have IL-1 activity. The means (and their standard errors) from triplicate thymocyte cultures are presented.

Topical 1.0% SM did not increase the amount of IL-1 released into the culture fluids by full-thickness human skin explants.

Table 18: Interleukin 1 activity of organ-culture fluids from rabbit skin topically exposed in vitro to 1.0% SM with and without added serum and/or added macrophages

Time in organ culture at 26 C	Macro-phages	SM		$MeCl_2$	
		No serum	Serum (10%)	No serum	Serum (10%)
3 hr (Exp. I)	-	4.6 ±0.5	5.0 ±0.2	5.7 ±0.8	12.3 ±1.1
	+	6.9 ±0.7	13.6 ±1.7	5.8 ±0.7	7.7 ±3.8
16 hr (Exp. I)	-	15.0 ±1.2	16.9 ±1.3	8.8 ±2.1	
	+	13.6 ±3.7	3.8 ±0.5	8.7 ±1.6	23.0 ±3.3
16 hr (Exp. II)	-	20.3 ±1.3	9.5 ±3.2	11.1 ±6.4	11.4 ±2.0
	+	10.3 ±6.0	19.2 ±2.5	28.8 ±4.1	12.2 ±0.8

Listed are the radioactive tritium disintegrations per min X 10^{-2} at a 1:16 dilution of dialyzed culture fluid (see Experimental Methods). Values above 10.0 (i.e., 1000 dpm) can be considered to have IL-1 activity. The means and their standard errors from triplicate thymocyte cultures are presented.

Table 19: Chemotaxins for granulocytes (PMN) and macrophages (MN) in culture fluids (CFs) from full-thickness, 1.0-cm^2 human skin explants topically exposed in vitro to SM, nitrogen mustard (HN2), or methylene chloride ($MeCl_2$)

Cell type	Time after SM or HN2 exposure during which CFs were collected	Number of people donating skin specimens	Chemotactic activity of CFs percent of controls (E/C X 100%)*	P values**
PMN	3-4 h	4	88 ±7%	N.S.***
	20 h	10	60 ±17%	P <0.02
MN	3 h	4	60 ±19%	P = 0.06
	20 h	9	44 ±16%	P = 0.005

* Chemotactic activity of culture fluids from explants exposed to SM or HN2 (E) divided by the chemotactic activity of culture fluids from explants exposed to the diluent $MeCl_2$ (C) times 100.

** The one-tailed, matched-paired Student's *t* test was used.

*** N.S. = not (statistically) significant.

For these assays, the culture fluids from 3 to 12 explants from a given human skin were pooled and assayed in triplicate.

About 60% of the assays were made with 1.0% SM, and 40% with 5.0% HN2. The mustards decreased the chemotaxins released from the explants. The original data from which this table was derived are presented as Tables 22 and 23 (on pp. 53 and 54) in the Addendum.

Table 20: C3a and C5a present in culture fluids (CFs) from full-thickness, 1.0-cm^2 human skin explants topically exposed in vitro to SM or $MeCl_2$

Time after SM or $MeCl_2$ exposure during which CFs were collected		C3a content (average and range)	C5a (chemotaxis equivalent)
3 hr	$MeCl_2$	64 ±5 ng/ml	0
	1% SM	70 ±6 ng/ml	0
17 hr	$MeCl_2$	115 ±13 ng/ml	11.7 $X10^{-10}$ M
	1% SM	142 ±51 ng/ml	2.7 $X10^{-10}$ M

The culture fluids were cleared by centrifugation and frozen until they were assayed for C3a and C5a (75). All of the explants came from the same human skin specimen. The culture fluids were assayed for C3a in duplicate, and for C5a just once. These few assays indicate that SM applied topically to full-thickness human skin explants produced no significant change in the C3a or C5a content of the culture fluids.

ADDENDUM

Table 21: The number of paranuclear vacuoles in SM-exposed and control groups after incubation at 4 C or 36 C for 24 hr

Skin sample no. and source of normal skin	Days in storage at 4 C or 36 C	Number of paranuclear vacuoles in a central 1.0-cm tissue section across the explant after 24-hr incubation at 4 C or 36 C					
		Controls			1.0% SM		
		storage type	toxicant type	total	storage type	toxicant type	total
#302 leg, white male age 45	1 day 36 C	38	13	51	101	440	541
#316 Breast, white female, age 68	1 day 36 C	350	28	378	54	410	464
#427 Breast, black female, age 21	1 day 36 C	18	7	25	155	1740	1895
#428 Neck, white female, age 57	1 day 4 C	19	8	27	70	720	790
#601 Face, white female, age 47	1 day 36 C	14	2	16	65	670	735
#620 Breast, white female, age 50	1 day 36 C	46	17	63	25	290	315
#710 Neck, white female, age 59	1 day 36 C	57	26	83	54	610	664
#913 Neck, white female, age 55	1 day 4 C	49	21	70	92	560	652
#918 Neck, white female, age 47	1 day 4 C	30	14	44	63	850	913

This table extends the series in Table 1 with 1.0% SM (instead of 0.2% SM). As in Table 1, much variation occurs among the human skin specimens, and the highest number of vacuoles occurred in a specimen of breast skin from a young woman. The neck skins were from face lift operations.

ADDENDUM

Table 22: Chemotaxins for PMN in culture fluids (CFs) from full-thickness, 1.0-cm^2 human skin explants topically exposed *in vitro* to SM, HN2, or $MeCl_2$

Number of human skin specimens and treatment	Time after SM or HN2 exposure during which CFs were collected	Chemotactic index of PMN				Chemotactic activity of CFs percent of controls (E/C X 100%)
		1	2	3	mean	
No. 1 $MeCl_2$ 1% SM	20 h	614	374	475	488	
		131	91	192	138	28%
No. 2 $MeCl_2$ 1% SM	20 h	466	594	192	417	
		224	316	202	247	59%
No. 3 $MeCl_2$ 1% SM	20 h	204	363	557	375	
		81	110	77	89	24%
No. 4 $MeCl_2$ 1% SM	20 h	319	104	140	188	
		69	35	41	48	26%
No. 5 $MeCl_2$ 1% SM	20 h	124	130	165	140	
		198	120	285	201	144%
No. 6 $MeCl_2$ 1% SM	4 h	43	24	31	33	
		40	22	21	28	85%
$MeCl_2$ 1% SM	20 h	278	315	399	331	
		100	43	24	56	17%
No. 7 $MeCl_2$ 5% HN2	20 h	181	155	60	132	
		60	114	58	77	58%
No. 8 $MeCl_2$ 5% HN2	3 h	17	62	52	44	
		43	52	47	47	107%
$MeCl_2$ 5% HN2	20 h	209	143	104	152	
		48	23	58	43	28%
No. 9 $MeCl_2$ 5% HN2	3 h	77	98	106	94	
		50	80	72	67	71%
$MeCl_2$ 5% HN2	20 h	39	62	61	54	
		121	105	48	91	169%
No. 10 $MeCl_2$ 5% HN2	3 h	72	59	53	61	
		57	55	47	53	87%
$MeCl_2$ 5% HN2	20 h	166	99	67	111	
		49	59	37	48	43%

ADDENDUM

Table 23: Chemotaxins for macrophages in culture fluids (CFs) from full-thickness, 1.0-cm^2 human skin explants topically exposed in vitro to SM, HN2, or $MeCl_2$

Number of human skin specimens and treatment		Time after SM or HN2 exposure during which CFs were collected	Chemotactic index of macrophages				Chemotactic activity of CFs percent of controls (E/C X 100%)
			1	2	3	mean	
No. 1	$MeCl_2$ 1% SM	20 h	21 5	-- 4	-- 6	21 5	24%
No. 2	$MeCl_2$ 1% SM	20 h	-- 12	-- 31	-- --	-- 22	
No. 3	$MeCl_2$ 1% SM	20 h	80 15	83 16	49 18	71 16	23%
No. 4	$MeCl_2$ 1% SM	20 h	75 20	58 20	46 16	60 19	32%
No. 5	$MeCl_2$ 1% SM	20 h	26 66	26 53	35 29	29 49	169%
No. 6	$MeCl_2$ 1% SM	4 h	29 11	26 11	28 7	28 10	36%
	$MeCl_2$ 1% SM	20 h	76 18	105 11	99 8	93 12	13%
No. 7	$MeCl_2$ 5% HN2	20 h	114 51	108 76	132 44	118 57	48%
No. 8	$MeCl_2$ 5% HN2	3 h	9 0	3 2	3 0	5 1	20%
	$MeCl_2$ 5% HN2	20 h	23 5	42 3	24 6	30 5	17%
No. 9	$MeCl_2$ 5% HN2	3 h	8 5	7 4	4 6	6 5	83%
	$MeCl_2$ 5% HN2	20 h	18 7	12 1	13 11	14 6	43%
No. 10	$MeCl_2$ 5% HN2	3 h	5 2	3 2	4 7	4 4	100%
	$MeCl_2$ 5% HN2	20 h	10 2	14 7	14 4	13 4	31%

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